Fish Screening Assay Discussion Paper
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Introduction

The Organisation for Economic Co-operation and Development (OECD) Validation Management Group for Ecotoxicity Testing (VMGeco) agreed to recommend to the OECD Task Force on Endocrine Disrupter Testing and Assessment (EDTA) and the Working Group of the National Co-ordinators of the Test Guidelines Programme (WNT) that an OECD Test Guideline be developed for a fish screening assay based on the Phase 1B validation trial protocol using vitellogenin (VTG) and secondary sex characteristics (SSC) as core endpoints. The objective of this assay, as recommended, is to detect estrogenic and androgenic agonists and aromatase-inhibiting substances. This agreement was conditioned upon the test guideline being able to accommodate additional endpoints (*e.g.,* fecundity and histopathology) once adequate data are made available to demonstrate their validity. The condition was needed because a minority view, which the U.S. held, was that the assay with only VTG and SSC would be inadequate to detect all known modes of action (*e.g.*, androgen receptor antagonism) and further limit the test guideline for other purposes, such as detecting reproductive toxicants through non-endocrine mechanisms.

The purpose of this paper is to briefly summarize the available data and present a rationale to support the U.S. contention that a broader suite of endpoints are ready to be included in the Fish Screening Assay Test Guideline. The U.S. asserts that the reliability of the additional endpoints is uniformly demonstrated for use in a screening assay, albeit some additional interlaboratory data are acknowledged will be needed with the standardized protocol to fully satisfy validation requirements. The data presented focus on the fathead minnow and include results from over sixty (60) individual studies encompassing twenty (20) chemicals, seventeen (17) laboratories, and sixteen (16) protocol variations (see Addendum 1). Eighteen (18) of these studies conducted in five (5) different laboratories were 21-day assays that included a suite of endpoints generally consistent with the proposed standardized protocol provided in Addendum 2. These studies effectively demonstrate the utility and reliability of this assay for its purpose in screening substances that potentially interfere with estrogen and androgen mediated processes. The summary of data presented in this paper is not intended to be exhaustive and detailed, only to emphasize that there is a fair and compelling body of information which is available that support the U.S. assertion for the fish screen with the fathead minnow. Data on medaka and zebrafish are not included because this would make the presentation more complex, but it should be understood that similar

supportive comparisons could be made for these species as well. It is expected that a detailed and exhaustive integrated summary validation report can be developed from the existing data sufficient to advance the protocol as presented in Addendum 2 for affirmation by a suitable peer review. That said, it is also unfortunately clear that an additional interlaboratory comparison is warranted with the standardized protocol to solidly demonstrate the reproducibility of the assay and this need will also be discussed.

Background

The OECD, in 1998, established the EDTA at the request of member countries as a Special Activity of the Test Guidelines Programme. The U. S. was one of the member countries who strongly encouraged this Special Activity due to its own legislative mandates and the recognized need to harmonize the development and validation of new and enhanced methods for evaluating endocrine disrupting chemicals (EDCs). The agreed focus of the EDTA, which is consistent with the U.S.'s and other member countries' interests, was on methods which could potentially cover the disrupting effects of chemicals on the reproductive system (e.g., estrogen agonists/antagonists and androgen agonists/antagonists) and on development (e.g., thyroid axis)(OECD 2004b).

In response to its own statutory obligations, the U. S. Environmental Protection Agency (EPA) implemented an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed to detect and characterize potential endocrine effects of environmental contaminants, industrial chemicals, and pesticides. The U.S. program is a two-tiered approach which employs a combination (e.g., battery) of *in vitro* and *in vivo* screens in Tier 1 for assessing the potential to affect endocrine-mediated processes and *in vivo* testing using two-generation studies in Tier 2 for characterizing dose-response and potential adverse effects. A fish assay in the Tier 1 screening battery was identified as an essential component. It is important because there is good evidence finfishes are being affected by EDCs in the field (Ankley and Giesy 1998; Crisp et al 1998). Additionally, estrogenic and androgenic controls in reproduction and development in fishes differ enough from those in higher vertebrates that screening limited to a single taxon (e.g., rodents) may not adequately identify potential EDCs across all vertebrate classes.

A Detailed Review Paper (DRP) has been completed which provides a thorough overview and scientific basis for a fish screening assay to be used in the detection of endocrine active substances (OECD 2004b). The purpose of the assay as expressed in the DRP is "to provide an indication of the potential of a chemical to behave as an endocrine-active substance on the organism studied

and thus to guide on whether additional testing will be needed to better characterize the potential endocrine disrupter". Further, the DRP states that it "is not designed to identify specific mechanisms of hormonal disruption (such as hormone receptor agonistic or antagonistic effects, or disturbance of hormone synthesis or metabolism)".

It is important that the purpose of the Fish Screening Assay is clearly articulated and understood for adequate protocol validation. In the EDSP, screening is defined as "the application of assays to determine whether a chemical substance or mixture may interact with the endocrine system" (US EPA 1998). The fish short-term reproduction assay is one of a coordinated battery of assays that support this purpose. The specific role established for the fish assay as part of the EDSP is to assess the potential for a chemical substance to affect estrogen- and androgen-mediated processes in this vertebrate class. "The test is designed as a short-term reproduction assay suitable for identifying chemicals that affect reproduction or, potentially, development through disruption of any number of pathways, including those controlled by estrogens and/or androgens." (US EPA 2002) By using intact mature and actively spawning fishes, the assay can assess the entire hypothalamus-pituitary-gondal (HPG) axis which directly affects these sex steroids. Essential endpoints included in the assay cover biochemical (VTG and steroids), tissue (gonad histopathology), and organism (SSC and fecundity) level responses influenced by these endocrine pathways. The assay has an added benefit of capturing the potential for a chemical substance to affect reproductive output through broader endocrine and non-endocrine mediated processes as well. This additional role for the assay has been accepted as integral to the assay purpose for EPA regulatory needs. This is consistent with what is also expressed in the DRP (OECD 2004b):

> "Endpoints should be reflective of specific disturbances at the (sub-) organism level and will be a combination of apical, histological and biochemical measurements. This is to establish a link between a response at the sub-organism level and a change observed at the organism level. Such a combination of measurements will also aid in the interpretation of test results and inform on the additional testing needs."

The screening assays in the EDSP were recommended (US EPA 1998) to capture all known modes by which the estrogen, androgen, and thyroid hormone systems can be affected. As stated, a screening assay is not intended to define a substance's mode of action or the specific biological mechanism/path with which it interacts toxicologically. Such an objective is more suited to a dedicated research program than to a simple screen. It should also be noted that a single chemical may also have multiple modes of action which thereby require that the screening assays be capable of detecting multiple modes of action,

although it is recognized that not all mode of actions (MOAs) have been yet recorded or examined for the endocrine system. A screening assay is intended only to provide suggestive information that an endocrine-mediated process is involved, and certainly not confirm it. It is therefore not necessary to resolve whether an effect to an endpoint of regulatory concern such as reproductive output is due to endocrine or non-endocrine mechanisms. The outcome of a screen which identifies an effect on reproductive output, for example, under existing U.S. pesticide regulations (40 CFR Part 158) will be to derive a dose-response relationship in a definitive trial regardless of the mechanism involved. However, the combination of endpoints within the assay and across the battery of assays employed does provide some information to corroborate whether an endocrine or non-endocrine process is more likely. It is this weight-of-evidence approach to the data interpretation that is critical for determining the need for further tier testing progression.

The standardized fish screening assay protocol, as appropriate to the fathead minnow (*Pimephales promelas*), which includes a comprehensive suite of endpoints and which is being advocated by the U.S. for the final steps of validation is presented as Addendum 2.

Chemicals employed in validation

Chemicals which have been employed in evaluation of the 21-day fish reproduction screen for fathead minnow (*Pimephales promelas*) are grouped by their predominant (anticipated) HPG MOA:

Androgen receptor agonists -
- Methyltestosterone
- 17β-trenbolone
- 17α-trenbolone

Androgen receptor antagonists -
- Flutamide
- Vinclozolin
- p,p'-DDE

Estrogen receptor agonists -
- 17β-estradiol
- 4-*tert*-pentylphenol
- Bisphenol A
- Methoxychlor

Steroid metabolism modulators -
- Fadrozole
- Fenarimol
- Ketoconazole
- Prochloraz
- Cadmium chloride

Uncertain modes of action -
- Atrazine
- Prometon
- Perfluorooctane sulfonate (PFOS)

Negative compounds -
- Potassium permanganate
- Perchlorate

Among these chemicals, those with possible multiple MOAs (based both on *in vivo* and *in vitro* analyses) include:
- Methoxychlor (estrogen receptor agonist/androgen receptor antagonist)
- Methyltestosterone (androgen receptor agonist/estrogen receptor agonist through aromatization)
- Fenarimol (aromatase inhibitor/estrogen receptor antagonist/androgen receptor antagonist)
- Prochloraz (multiple steroidogenesis enzyme inhibitor/androgen receptor antagonist)
- Ketoconazole (multiple steroidogenesis enzyme inhibitor)

The chemicals chosen to represent androgen receptor agonist modes of action in the fish screen include methyltestosterone, 17β-trenbolone, and 17α-trenbolone. Methyltestosterone is a synthetic derivative of testosterone which is aromatizable (to methylestradiol; Hornung et al 2004), and therefore may have estrogenic effects in addition to androgenic effects. 17-β and 17-α trenbolone are active metabolites of trenbolone acetate, a chemical used in food animal production that improves weight gain, and can be found in the environment. 17β-trenbolone has been shown to be highly androgenic in fathead minnow, resulting in masculinization and reduction of vitellogenesis in females (Ankley et al 2003). There is no evidence to suggest that trenbolone is aromatizable. Effects of androgen receptor agonists in fathead minnow include masculinization of SSC, reduced fecundity, impaired vitellogenesis, and pathological effects on the gonads. Potential histopathological changes associated with androgen agonists include suppression of oocyte maturation coupled with increased oocyte atresia and reduction in post-ovulatory follicles (Ankley et al 2001). Exposure to strong androgen receptor agonists may also manifest as lowered yolk deposition in

oocytes (Ankley et al 2003). In males, secondary sex characteristics may be magnified, and spermatogenesis may be inappropriately stimulated (Ankley et al 2001).

Androgen receptor antagonists, or anti-androgens, can exert feminizing effects on SSC. They also may affect spawning, gonadal development (especially testicular development), and gonadal histopathology including changes in spermatogenesis or changes in the ratio of spermatogenic cells. Potential effects of anti-androgens in the fathead minnow include effects on spermatogenesis and yolk deposition in oocytes (Makynen et al 2000), and increased oocyte atresia (Jensen et al 2004). Representative chemicals for androgen receptor antagonism used in the evaluation of the short-term fish reproduction assay include flutamide, vinclozolin, and p,p'-DDE. Flutamide and its 2-hydroxyflutamide metabolite are antagonists of the mammalian androgen receptor, and they bind to the receptor of the fathead minnow (Ankley et al 2004; Makynen et al 2000). Vinclozolin, a fungicide thought to promote Leydig cell tumors, and which exerts anti-androgenic influences on sexual differentiation in mammals, is thought to interfere at the level of the androgen receptor (Gray et al 1994). p,p'-DDE is a metabolite of the pesticide dichlorodiphenyltrichloro-ethane (DDT). Past studies in rodents suggest that p,p'-DDE may interfere with binding of endogenous androgens at the androgen receptor (Gray et al 1999; Kelce et al 1995, 1997).

The short-term fish reproduction assay also has the ability to detect estrogenic substances. The most active endogenous estrogen, 17β–estradiol, is necessary for the normal growth and development of the female sex organs, and in some species contributes to the development and maintenance of female secondary sex characteristics. Estrogens also influence the release of gonadotropins from the pituitary gland. Reported changes in fathead minnow due to estrogen exposure include SSC changes, sex ratio changes, VTG induction, steroid hormone changes, changes in fertility and fecundity, and histologic alterations in gonadal tissues (Lange et al 2001; Lattier et al 2002; Miles-Richardson et al 1999b; Parrott and Blunt 2005; Pawlowski et al 2004b; Sohoni et al 2001). Compounds that were used in the evaluation that represent estrogen receptor agonists include 17β-estradiol as a strong estrogenic substance, and 4-*tert*-pentylphenol, methoxychlor, and bisphenol A as weakly estrogenic compounds. Methoxychlor is a relatively non-persistent organochlorine insecticide registered for insect control in post-harvest applications in a variety of crops, foliar treatment, soil, and seed treatment. It has been shown to have estrogenic influences in several fish species, including induction of vitellogenesis (in males) and negative impacts on fecundity (Ankley et al 2001; Hemmer et al 2001; Kobayashi et al 2003; Versonnen et al 2004). Bisphenol A is a high-production chemical used to make polycarbonate plastic and epoxy resins. A

previous study in which fathead minnows were exposed to various concentrations of bisphenol A for up to 164 days reported VTG induction in males at an approximate exposure level of 160 µg/L. Higher exposure levels (640 µg/L) also significantly raised VTG levels in females and diminished egg hatching success (Sohoni et al 2001). Additional effects reported were a decrease in fecundity at exposure levels of 1280 µg/L. Four-*tert*-pentylphenol is a member of the alkylphenol family. It has been shown to demasculinize male fish and induce vitellogenesis (Gimeno et al 1998; Panter et al 2002). Estrogenic substances may also induce histopathologic changes of the gonads, including testicular oocyte formation, necrosis of testicular germ cells and spermatozoa in conjunction with hyperplasia and hypertrophy of Sertoli cells (Miles-Richardson et al 1999b), increased oocyte atresia (Ankley et al 2001) or alteration in oocyte maturation, and increased interstitial fluid (Lange et al 2001; Miles-Richardson et al 1999a). Pathologic changes may also occur in the kidney of males due to the ineffective removal of excessive vitellogenin from the blood.

Steroidogenesis is the production of the steroid hormones required for reproduction. In the male, steroidogenesis occurs in the Leydig cells of the testis and, in the female, this pathway is found in the follicle of the ovary. Steroidogenesis also takes place in the adrenal tissue. At the cellular level, a series of biochemical reactions are initiated upon stimulation of gonadotropin receptors located in the membranes of these cells. Activation of the luteinizing hormone/follicle stimulating hormone (LH/FSH) receptors initiates a series of enzymatic reactions that culminate in the biosynthesis of end-product hormones, *i.e.,* primarily testosterone (11-ketotestosterone) in males, and primarily estradiol in females. Due to its ability to detect changes in estrogens and androgens, the short-term fish reproduction assay can also detect interference in the steroidogenesis pathways. Potential findings in fathead minnow that are associated with steroidogenesis inhibition include suppression of vitellogenesis through decreasing serum estradiol levels, decreased numbers of post-ovulatory follicles, changes in the normal maturation processes of oocytes and spermatozoa, and effects on the ability of female fish to spawn (Ankley et al 2002).

Chemicals used in the evaluation that interfere with normal steroidogenesis include fadrozole, fenarimol, ketoconazole, prochloraz, and cadmium chloride. Fadrozole is a therapeutic chemical developed to treat estrogen-dependent diseases, including breast cancer. It is a competitive inhibitor of P-450 aromatase (CYP19) interfering with the conversion of C19 androgens to C18 estrogens. Fenarimol, ketoconazole and prochloraz are all conazole fungicides designed to inhibit fungal growth by blocking a cytochrome P-450 (CYP) enzyme that makes ergosterol. Although this particular CYP is not found in vertebrates, these compounds can affect CYP enzymes involved in

steroidogenesis such as P-450scc, lyase, and aromatase. This causes reductions in steroid synthesis. Prochloraz, for instance, may affect aromatization of testosterone to estrogen in addition to its effects on other CYPs (Ankley et al 2005a). Cadmium has been suggested as inhibiting testosterone production in Leydig cells of male gonads, in addition to progesterone, testosterone, and estradiol production in ovaries (Leblond and Hontela 1999; Piasek and Laskey 1994). Potential effects of steroidogenesis inhibitors in fathead minnow include impaired vitellogenesis, pronounced oocyte atresia and suppression of oocyte maturation, and ultimately a reduction in fecundity (Ankley et al 2002; Ankley et al 2005a).

Several chemicals with uncharacterized modes of action were also used to evaluate the fish screen. These chemicals include atrazine, prometon, and perfluorooctanesulfonate (PFOS). Atrazine is a widely used chlorotriazine herbicide. Because of its worldwide agricultural usage, the toxicity of atrazine has received considerable study. The suspected endocrine mode of action is considered to be at the neuroendocrine level (hypothalamus-pituitary axis), although this is based primarily on mammalian studies (Cooper et al 1999). Prometon is a structurally-related methoxytriazine herbicide. PFOS is a stable metabolite of fluorinated surfactants and has been found in myriad wildlife species (Giesy and Kannan 2001). There is some evidence that structurally similar chemicals to PFOS can alter plasma concentrations of both androgens and estrogens in fathead minnow (Oakes et al 2004). Due to the relative scarcity of information regarding the effects of atrazine, prometon, and PFOS in fathead minnow, these chemicals were chosen to evaluate the ability of the fish screen to detect effects of uncharacterized potential endocrine modulators.

To test the specificity of the fish screen, two chemicals were used that are not known to be disruptive of reproductive endocrine processes. These two chemicals include potassium permanganate and perchlorate. Potassium permanganate is an oxidizing agent that reacts with organic matter and is used to treat several common fish diseases. Perchlorate is a by-product of rocket fuel production widely found in groundwater; it is known to competitively inhibit iodine uptake by the thyroid gland (Wolff 1998). Decreased iodine uptake alters thyroid gland function and lowers production of thyroid hormones. The goal of testing potassium permanganate and perchlorate was to determine whether general, biological stress responses affect the estrogen and androgen related endpoints in the fish screen.

Endpoints

Vitellogenin

Vitellogenin is a female-specific yolk-precursor protein that is primarily regulated through the interaction of β-estradiol and the estrogen receptor (Kime 1998; Sumpter and Jobling 1995). It is expressed in sexually mature females of oviparous species and is required for development and maturation processes of oocytes. In males, the VTG genes are normally inactive; however, they retain the ability to produce VTG in response to estrogenic compounds, and in fathead minnow, induction by exposure to estrogen receptor agonists has repeatedly been demonstrated (Brodeur et al 2005; Hansen et al 1998; Harries et al 2000; Korte et al 2000; Kramer et al 1998; Lattier et al 2002; Panter et al 1998; Parks et al 1999; Schmid et al 2002). In addition, it has been shown that exposure of female fathead minnow to androgens can suppress vitellogenesis, potentially by depressing the endogenous androgens which are converted to estradiol (Ankley et al 2003; Miracle et al 2006).

There are several advantages to the use of VTG as an endpoint in the fish screen. One advantage is that it is primarily controlled through estrogen interaction with the estrogen receptor, and hence is directly related to a mechanism of concern (Korte et al 2000; Mori et al 1998). As mentioned above, it may also respond secondarily to androgenic compounds through suppression of natural androgens and subsequent reduction in endogenous estrogens. This secondary mechanism may also manifest when fish are exposed to a steroidogenesis (e.g., aromatase) inhibitor due to the impaired ability to adequately produce endogenous estrogens. Another advantage to the vitellogenin endpoint is the increasing commercial availability of ELISA kits that are specific to fathead minnow vitellogenin (Jensen et al 2006).

Because the endocrine system is integrative, by definition, and the mechanisms of vitellogenesis are not fully characterized, there are some issues to consider in the interpretation of vitellogenin results. First, vitellogenesis relies on the presence of adequate numbers of functional estrogen receptors in hepatocytes, as well as a clinically normal liver capacity. Hence, there is potential for changes in vitellogenesis to occur secondarily if compounds affect the number of estrogen receptors, or the ability of the liver to produce proteins. There is some evidence that cortisol may affect vitellogenesis through reduction of expression of the estrogen receptor in trout (Lethimonier et al 2000), however in fathead minnow, others have demonstrated that cortisol may potentiate, rather than suppress, vitellogenesis (Brodeur et al 2005). Also, in theory, compromised liver capacity could affect the ability of a fish to synthesize

vitellogenin. While it is likely that if a compound is toxic enough to compromise liver capacity, other aspects of the assay will also be affected and the fish would likely show clinical signs of disease, the possibility of liver toxicity should not be excluded as a potential cause of decreased vitellogenesis. Prolactin and thyroid hormones have also been postulated to influence vitellogenin synthesis (Sumpter and Jobling 1995).

Histopathology

Toxicologic histopathology is used to identify and describe morphologic differences between unexposed (negative control) and compound-exposed animals. Histopathology is a necessary endpoint in the fish screen assay because it is a direct evaluation of the reproductive organs of interest, and histopathologic changes express the integration of several molecular, cellular, and physiologic processes. In addition, it provides insight on the potential reproductive impacts of chemical disruption, and it is useful to confirm changes in other endpoints of the assay. Other recognized benefits of histopathology are the ability to assess the general health of test populations, and the ability to identify causes of morbidity and mortality not associated with test compounds or reproductive endocrine activity.

Histopathology has served as a fundamental endpoint in the mammalian toxicology testing arena. Even though it has not received as much attention in other vertebrate taxa, it has proven to be useful in understanding and assessing the effects of endocrine active substances in fish (Ankley et al 2001, 2002, 2003, 2004; Ankley and Johnson 2004; Van der Ven et al 2003; Wester and Canton 1991; Wester and Van der Ven 2000;) and is considered an integral part of the fish screen. In addition, much work has been accomplished that characterizes the normal histology of gonads in healthy fathead minnow and the typical changes that might be expected in gonads in response to endocrine disruptor exposure (Leino et al 2005).

Histopathology has been useful and informative during the validation effort for the fish screen. For example, during the OECD Phase 1A analysis of the fish screen validation effort, male and female fish were kept separated and gonadal response to exposure to EDCs was confounded by ovarian pathology. In recognition of the concern raised about the ovarian response of treated and untreated fish in the Phase 1A trial, the pathologists suggested that the forced non-spawning conditions were responsible and these stressed conditions created a high rate of atresia in ovaries of control animals. They recommended that the fish be allowed to spawn in OECD Phase 1B tests by housing the males and female fish in the same aquaria to remove this confounding factor and improve the readability of gonad slides. Lothenbach et al. (2005) investigated the gonad

histology of spawning versus non-spawning medaka and confirmed the hypothesis of the histopathology expert group. This example clearly demonstrates the utility of histopathology as it relates to determining the general health of the test population and identifying confounding factors that might affect the performance of the endpoints in the fish screen.

The gonad is the primary organ of reproduction and, as such, will reflect substantive disturbances to the HPG axis. The gonad also encompasses the many physiologic responses made by an endocrine-active substance that affects the reproductive system. It is more general than a biochemical measurement, and it changes in response to multiple stressors, both chemical and non-chemical. The fine structure of gonadal tissue provides tangible and verifiable evidence for the health or dysfunction of the organ.

For histopathological analysis to be most useful in reproductive screening tests, it is important to ensure maximum control of the subjective nature of the interpretations, and to limit the interpretation to repeatable results that can be verified by different investigators. In support of improving diagnostic consistency and reducing bias, an intensive effort has been made to standardize reading practices, diagnostic terminology, and severity grading approaches, which is documented in the draft guidance document on fish histopathology (OECD 2004a). For an in-depth description of the histopathology standardization efforts to support the validation effort for the fish screen, please refer to the OECD final report for pathologists post-Heidelberg, and the OECD guidance document for fish histopathology (OECD 2004a; OECD 2004c). Briefly, lists of primary and secondary diagnoses (Tables 1 and 2) were established which standardize the description of criteria used for diagnostic purposes, and that minimize the time and reporting effort required by pathologists reviewing studies. Each of the four primary diagnoses is directly relevant to endocrine function within the gonads and pathologists would routinely assess these diagnoses. Secondary diagnoses will only be noted if present.

Table 1. Histopathological Primary Diagnoses

Primary Diagnoses	
Males	Females
Increased proportion of spermatogonia	Increased oocyte atresia
Presence of testis-ova	Perifollicular cell hyperplasia/hypertrophy
Increased testicular degeneration	Decreased yolk formation
Interstitial (Leydig) cell hyperplasia/hypertrophy	Gonadal staging

Table 2. Histopathological Secondary Diagnoses

Secondary Diagnoses	
Males	Females
Decreased proportion of spermatogonia	Interstitial fibrosis
Increased proteinacesou fluid withing testicular vessels or interstitium	Egg debris in the oviduct
Asynchronous gonad development	Granulomatous inflammation
Granulomatous inflammation	Decreased post-ovulatory follicles

A severity grading system was also established to allow pathologists to take into account discrete, spatial, and global pathologic changes in the gonads on a 5-point scale. Each severity grade is described in the guidance document, and pathologists can score lesions in comparison to controls, or in comparison to "normal" gonad histology based on the pathologists' experience.

It has been discussed repeatedly and determined that the appropriate reading practice to employ for the fish screen is one that is non-blinded initially, with knowledge of control and treatment groups, in accordance with guidance from the Society of Toxicologic Pathology (Crissman et al 2004). Informal blinded re-evaluation is then performed to confirm diagnoses.

There is some concern that histopathology is a time-consuming and expensive endpoint for a screening assay. Standardization of histopathology, as described above, greatly reduces the time and cost associated with this endpoint. In addition, there may be more costly consequences of not performing histopathology as part of the fish screen. Besides being a sensitive indicator of endocrine dysfunction, histopathology confirms that the test population is healthy and responding normally. Confounding factors that can alter the molecular, cellular, and physiologic responses that affect the fish, and that might influence the outcome of the assay, can be detected using histopathology. Also, histopathology may decrease ambiguity when fish are exposed to chemicals with unknown modes of action, reconcile unexpected results from other endpoints and hence, may reduce the likelihood that assays must be run multiple times in such instances.

Fecundity

Fecundity is defined as the capacity to produce offspring and very broadly encompasses not only egg production, but fertility and hatching. Specifically for this assay, fecundity is measured by the total number of eggs, number of spawns, and number of eggs per spawn. Fertility is considered separately and recorded as the number of fertile and infertile eggs or proportion of fertile eggs. This determines the numbers of potential offspring. Fecundity is

a relevant endpoint for the fish screen because the definitive measure of reproductive success is the number of surviving progeny that carry genes from the parents, and who will in turn reproduce successfully (Kime 1998). It has been recommended that for the purposes of ecological risk assessment and to determine the effects of toxicants on reproductive fitness and population-level impacts, a measure of reproduction should be incorporated into the fish testing requirements (Suter et al 1987).

Fecundity as an endpoint is important to the fish screen because the number of eggs spawned is mediated, in large part, by the endocrine system. Changes in hormonal equilibrium could decrease the numbers and quality of eggs spawned. Fecundity can also be affected by gonadal development. It can be increased by recruitment of additional oocytes into vitellogenesis or it can be reduced by atresia (Hunter and Macewitz 1985). Certain EDCs can affect lifetime fecundity, survival of offspring to adulthood, and the fertility of the offspring.

Some advantages of the use of fecundity as an endpoint in the fish screen are that it can be collected non-invasively with minimal effort and does not require additional animal use. Fertility data can be collected easily at the same time egg counts are made, with minimal effort or time necessary when using fathead minnow. Fecundity data, when combined with gonadal histopathology, provides a good indicator of reproductive health of the fish. The biological relevance of fecundity far outweighs other potential endpoints, as it demonstrates potential population-level effects (Miller and Ankley 2004). Active spawning (egg production) can be used effectively as an acceptance or performance criterion of parental control fish for the study to ensure that fish are in an acceptable reproductive state for the duration of the study. Therefore, if egg production is already being collected for a portion of the fish used in the study, it could easily be collected for the fish used in the exposures in addition to those in the controls. Even though fecundity can be influenced by many chemical and non-chemical factors, its inclusion as an endpoint ensures that more endocrine effects will be caught, hence decreasing the incidence of false negatives. An added benefit of fertility determinations in conjunction with the fecundity endpoint is that this is an indication of male reproductive function (sperm quality).

Without question, fecundity represents the principal integrative endpoint in the fish short-term reproduction assay. The endpoint is easily and unambiguously collected from actively spawning fishes. Egg production data are needed to establish the acceptable spawning condition of test fish prior to exposure, and to establish acceptable control production during exposure. Reduced egg production has been demonstrated to be a sensitive response to exposure to known endocrine active substances including estrogen receptor

agonists, androgen receptor agonists, androgen receptor antagonists, and steroidogenesis inhibitors. It is also ethically incumbent to collect all available data practical from animals used in testing, especially where this information in one test will reduce the likelihood that repeated or additional tests would be necessary.

Secondary Sex Characteristics

Exposure of fish to chemicals with androgenic or estrogenic activity may result in degrees of abnormal sexual differentiation or function (Kime 1998). Certain characteristics particularly relevant to the fathead minnow in screening of EDCs include body color (*i.e,* light or dark), coloration patterns (*i.e.,* presence or absence of vertical bands), body shape (*i.e.*, shape of head and pectoral region, distension of abdomen), and specialized secondary sex characteristics (*i.e.*, number and size of nuptial tubercles, size/weight of the dorsal nape pad and ovipositor (US EPA 2002).

Secondary sex characteristics are used in the start of the screen to identify sexually mature fish to be used in the assay. General observations of physical appearance of adults and alterations of secondary sex characteristics (*i.e*., nuptial tubercles, dorsal nape pad, darkened coloration in males; distended abdomen and swollen ovipositor in females) can be made daily over the duration of the study and also at the conclusion of the screen.

The adult fathead minnow is sexually dimorphic, with males and females readily distinguishable from one another when in breeding conditions (US EPA 2002). Sexually mature males develop large nuptial tubercles on the snout and a dorsal pad that extends as a narrow band from the nape to the dorsal fin. Nuptial tubercles are "visible external horny outgrowths on the surface of the head" and dorsal nape pads are "soft enlargements of flesh on top of the head that extend onto the back of the fish to, or near, the anterior margin of the dorsal fin" which can be removed and weighed as an indication of effects of androgen agonists or antagonists, as well as estrogenic compounds (US EPA 2002; K. Thorpe, University of Exeter, personal communication). Males' bodies become black on the sides except for two wide light-colored vertical bars (US EPA 1987). However, females' bodies do not usually change in color or morphology, but they do develop a fleshy ovipositor that can be used to identify juvenile males from females (Flickinger 1969). Ovipositors are "urogenital structures present in females for egg production" (US EPA 2002). Females could also develop a dorsal pad which can be quantitatively measured in weight to assess androgen exposure.

Some EDCs can cause abnormal occurrence of certain secondary sex characteristics in the opposite sex. In the fathead minnow, external morphological features are under the control of sex steroids and therefore could be affected by estrogen or androgen receptor agonists or antagonists (Ankley et al 2001; Ankley et al 2003; Harries et al 2000; Lange et al 2001; Miles-Richardson et al 1999a; Smith 1974). Androgens can elicit certain biomarker sex characteristics in females whereas estrogens, anti-androgens, and steroidogenesis inhibitors suppress them in males. For example, androgen receptor agonists, such as methyltestosterone, can cause female fathead minnows to develop nuptial tubercles (Ankley et al 2001; Smith 1974) and/or dorsal nape pads (K. Thorpe, University of Exeter, personal communication). Also, some estrogen receptor agonists may decrease the number or size of nuptial tubercles in males (Harries et al 2000; Lange et al 2001; Miles-Richardson et al 1999a).

Some of the advantages of the use of secondary sex characteristics as endpoints in the fish screen with the fathead minnow are that these endpoints are biologically relevant and have been relatively reproducible. OECD and U.S. EPA data support consistent and reproducible results with potent androgens and estrogens. Male secondary sex characteristics provide the fish screen with indicative androgenic/antiandrogenic endpoints that may not be observed with other endpoints. Some of the weaknesses of using secondary sex characteristics as endpoints are that the sensitivity and specificity to certain MOAs are unknown. Although these characteristics are relatively easily measured, not all of them are as quantitative as some other endpoints. Also, some alterations to physiology that manifest in morphological changes may not appear in the short duration of the fish screen assay. In December 2005, the VMG-Eco group of the OECD advised that secondary sex characteristics in the fish screen were adequately developed in the fathead minnow and medaka for inclusion in the assay (OECD 2005).

Sex steroids

An endpoint which should be considered as an option in the protocol is the measurement of plasma sex steroid concentrations. A number of studies have been conducted documenting sex steroid levels in the fathead minnow under normal conditions and after exposure to endocrine-active compounds (Ankley et al 2001, 2003, 2005a; Giesy et al 2000; Jensen et al 2001; Jensen et al 2004; Makynen et al 2000) and this endpoint is included as a core endpoint in the EPA (2002) fish screening assay description.

Steroids measured in these studies include estradiol and testosterone and, occasionally, the fish-specific androgen 11-ketotestosterone. In terms of overall alterations in endocrine function (and reproductive success) steroid

concentrations tend to reflect changes in other endpoints (*e.g.,* changes in VTG or fecundity), but offer additional "value added" in two regards, as: (a) additional information in a weight-of-evidence analysis for identifying a chemical as endocrine-active, and (b) providing insights as to mechanisms associated with responses. For example, through analysis of steroid profiles it is possible to ascertain specific aspects of steroidogenesis that are affected in fathead minnows exposed to different model chemicals (Ankley et al 2002; 2005a).

Reported results

Available data are only briefly summarized in the body this paper. The current intent is to provide a simple overview of the studies supporting the assertion that the broader suite of endpoints are demonstrated to be reliable. The data are not critically analyzed in detail as would be expected in an integrated validation report. The tables in this section address four core endpoints (histopathology, VTG, SSC, and fecundity). Just those tests were included where mature fathead minnow and histopathology and/or fecundity endpoints were reported. Data were considered comparable whether a spawning group (*e.g.,* 2 males and 4 females) or a spawning pair (*e.g.,* one male and one female) was employed. The tables indicate only which endpoints were notably affected by the test compound. Histopathology findings are listed by the predominant diagnosis(ses) that were consistent among the laboratories reporting. The figures provide selected graphs and images from the studies to illustrate examples of endpoint responses. The listing of "additional diagnoses" (in conjunction with histopathology) notes that other pathologies were observed that may not have been specifically consistent across studies. Details of the specific pathologies reported can be found in Addendum 1. Addendum 1 also provides a more complete, though still limited, tabulation of the variety of tests completed with fathead minnow in the development of the fish screening assay.

Three androgen agonists have been investigated with a fathead minnow short-term reproduction assay comparable to the standardized protocol presented in Addendum 2. A summary of the findings for the core endpoints from these investigations are presented in Tables 3 – 5. All four endpoints were affected by the androgen agonist β-trenbolone in both laboratories which performed the assays (Table 3). The responses were also similar in both 14-day and 21-day versions of the assay performed by Battelle (2003a) indicating that, at the concentrations tested, a fairly rapid response time occurs for a potent androgen. The two laboratories investigating another androgen methyltestosterone also reported generally consistent findings on the four core endpoints (Table 4). Both laboratories reported an estrogen-like increase in VTG in both sexes at the same concentrations. Methyltestosterone, unlike β-

trenbolone, is aromatizable, which would explain the observed induction of VTG in males and females for this compound. A third androgen agonist (α-trenbolone) also affected all three endpoints (VTG, SSC, fecundity) which were included in the study observations (Table 5). All three androgens affected plasma sex steroid concentrations in the fish in a fashion consistent with feedback inhibition of steroid production (Ankley et al 2001, 2003; Jensen et al 2000b).

Table 3. β-Trenbolone

Endpoints	(Ankley et al 2003)	(Battelle 2003a) (21d)	(Battelle 2003a) (14d)
Histopathology			
Male			
additional diagnoses	+	+	+
Female			
increased oocyte atresia	+	+	+
additional diagnoses	+	+	+
Vitellogenin			
Male	+	-	-
Female	+	+	+
Secondary sex characteristics			
Male	-	-	-
Female	+	+	+
Fecundity	+	+	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 4. Methyltestosterone

Endpoints	(Ankley et al 2001)	(Pawlowski et al 2004a)
Histopathology		
Male		
additional diagnoses	+	NR
Female		
increased oocyte atresia	+	+
additional diagnoses	+	-
Vitellogenin		
Male	+	+
Female	+	+
Secondary sex characteristics		
Male	+	-
Female	+	+
Fecundity	+	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 5. α-Trenbolone

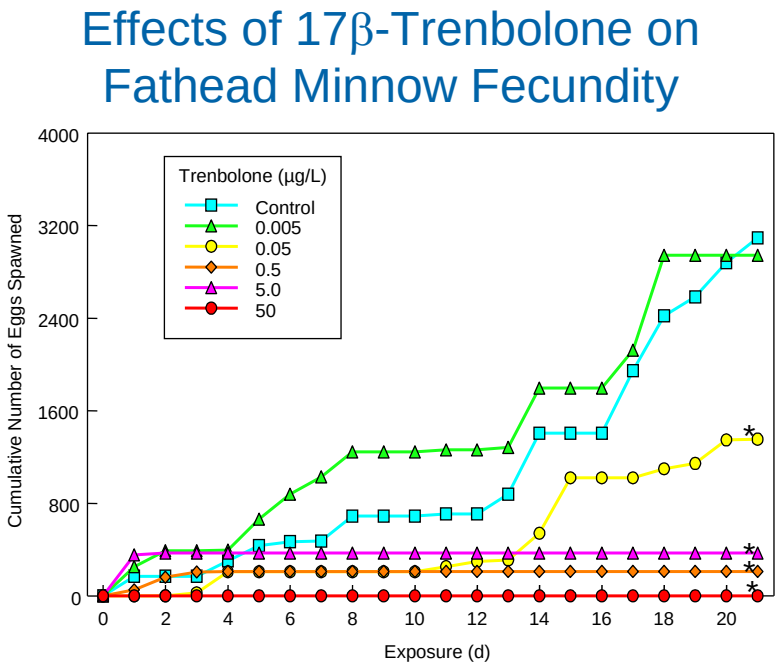
Endpoints	(Jensen et al 2006)
Histopathology	
Male	NR
Female	NR
Vitellogenin	
Male	-
Female	+
Secondary sex characteristics	
Male	-
Female	+
Fecundity	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

The OECD Phase 1A was conducted with a non-spawning version of the fish screening assay in four laboratories. 17β-trenbolone was one of two compounds evaluated in this initial interlaboratory trial. The 17β-trenbolone was observed to induce male SSC in fathead minnow (tubercles) and medaka females (anal fin papillary processes), but not zebrafish, and also decreased VTG in the females in all three species. However, gonadal histopathology results were seemingly compromised by high incidence of abnormalities in controls probably caused by "forced" cessation in spawning (Lothenbach et al 2005).

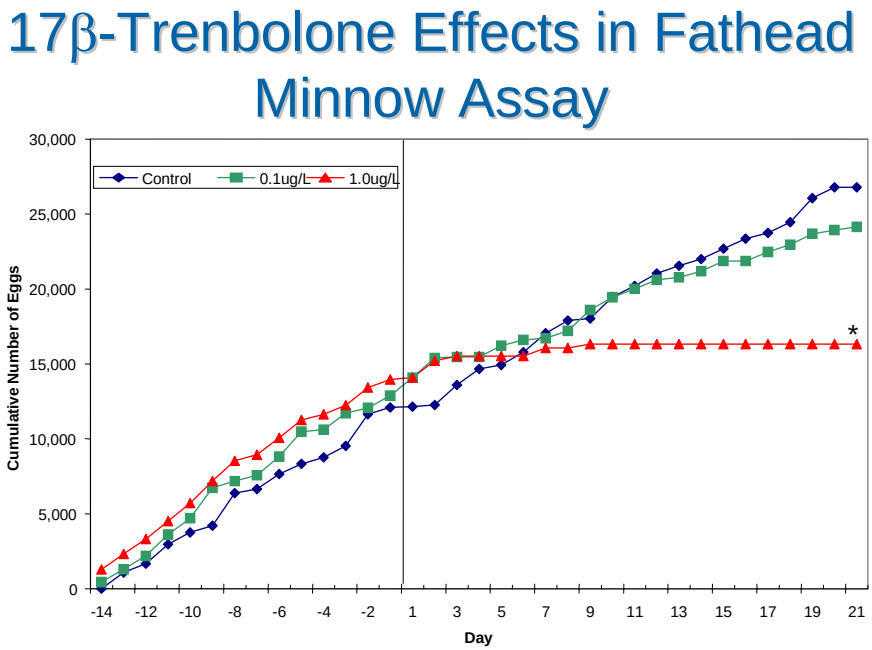
Overall, fecundity and SSC appear to be quite sensitive endpoints for androgen agonists (Figures 1-4). Fecundity responds quickly to an androgen agonist exposure, as is consistently seen across laboratories (Figures 1, 2, and 4). Androgen-characteristic masculinization of females is visibly evident in appearance of male nuptial tubercles (*e.g.,* Figure 3).

Figure 1.



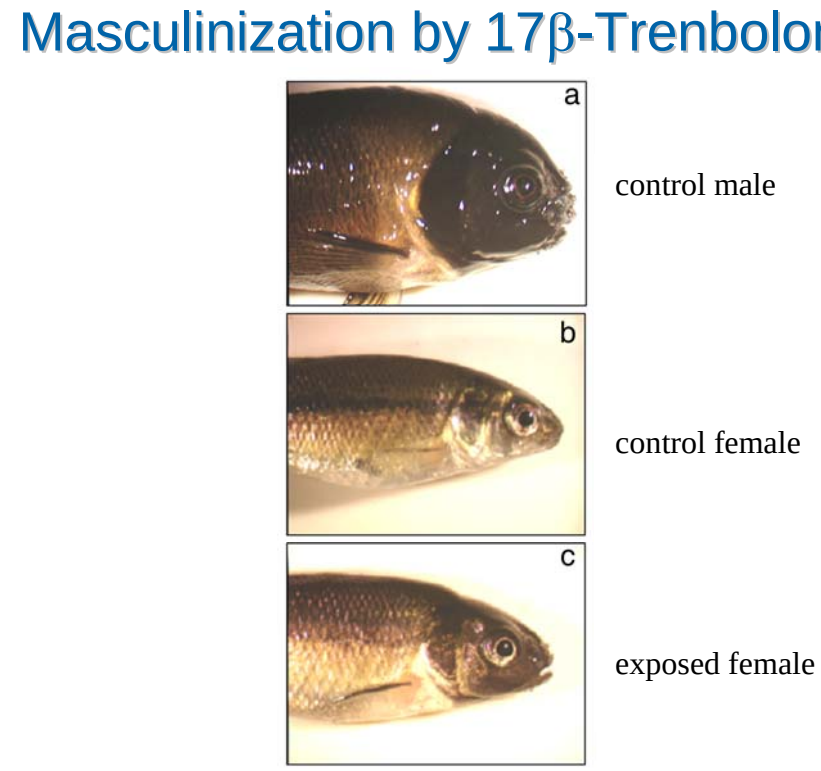
Ankley et al. (2003) Environ.Toxicol. Chem.

Figure 2.



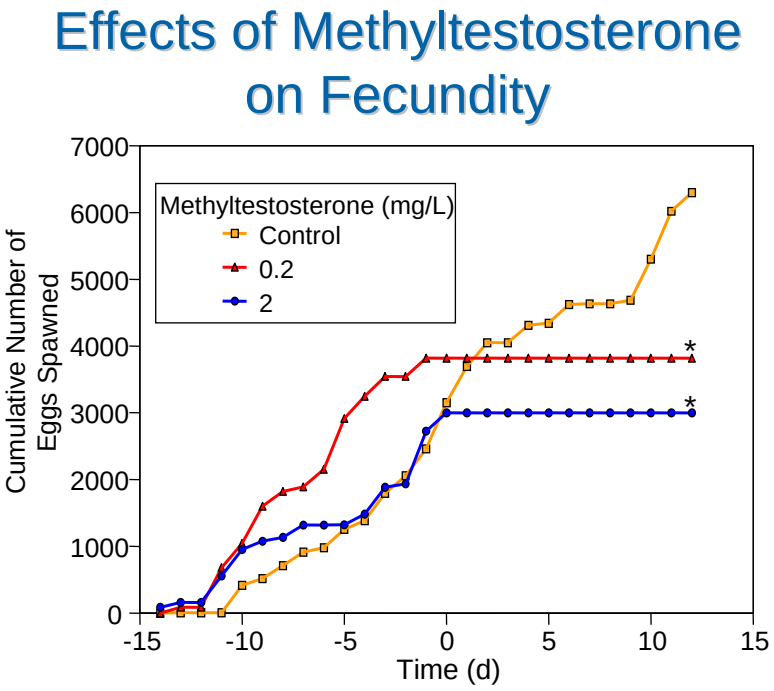
Battelle (2003a)

Figure 3.



Ankley et al. (2003) Environ.Toxicol. Chem.

Figure 4.



Ankley et al. (2001) Environ.Toxicol. Chem.

Fecundity also seems to be the most consistent endpoint responding to the androgen receptor antagonist flutamide (Table 6 and Figure 5). In the OECD Phase 1B trials (OECD 2006), the spawning configuration employed for fathead minnow (5 male:5 female per replicate) was sub-optimal and resulted in poor spawning in the control fish. In spite of this confounding factor, fecundity was still observed to be reduced in treated groups, which is consistent with the studies where spawning was acceptable (*e.g.*, Jensen et al 2004).

Table 6. Flutamide

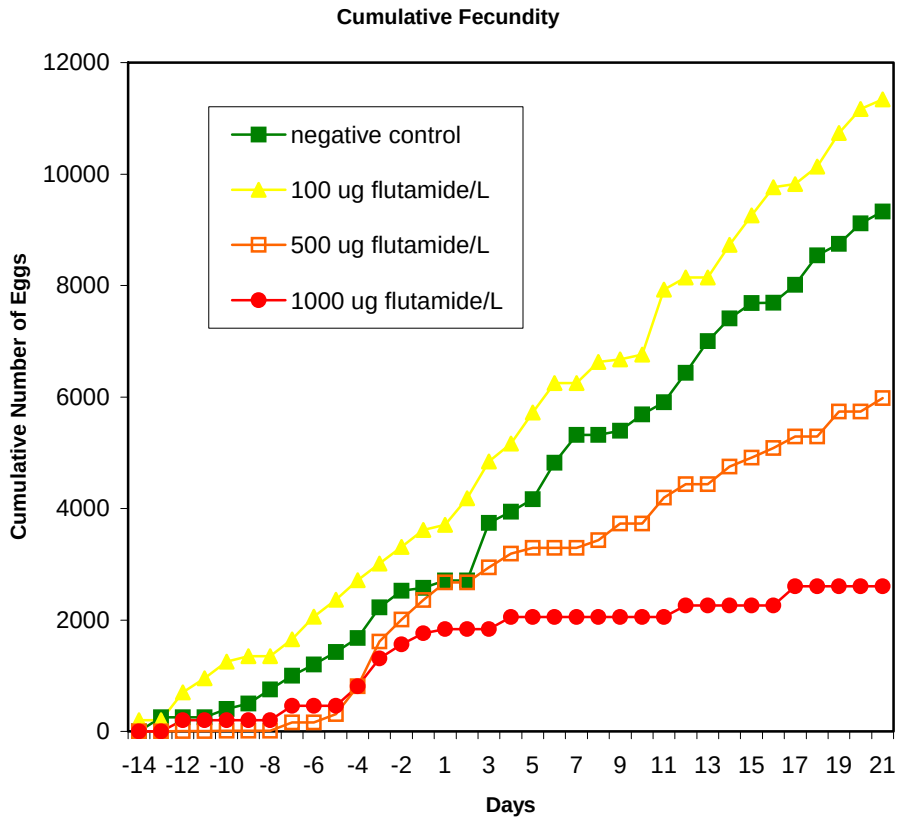
Endpoints	(Jensen et al 2004)	(Battelle 2003a) (21d)	(Battelle 2003a) (14d)	(Battelle 2006) (a)	(Battelle 2006) (b)	(OECD 2006) Lab 9	(OECD 2006) Lab 14	(OECD 2006) Lab 13
Histopathology								
Male								
additional diagnoses	+	-	-	+	-	-	+	-
Female								
increased oocyte atresia	+	+	+	+	-	-	-	+
additional diagnoses	+	-	-	-	-	-	-	-
Vitellogenin								
Male	+	-	-	-	-	-	-	-
Female	+	-	-	-	-	-	-	-
Secondary sex characteristics								
Male	-	-	-	+	+	-	-	-
Female	-	-	-	-	-	-	-	-
Fecundity	+	+	+	+	?	+	+	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
? = statistical reanalysis underway
NR = not reported, endpoint not included in study

Flutamide was also observed to substantially impair fertility in one study (Figure 6). Tubercle score and morphology were not significantly affected by flutamide. However, fatpad weight which was measured in two studies (Battelle 2006) was decreased by flutamide. Although histopathology was inconsistent in detecting flutamide responses, this could have been influenced by protocol deviations and by the fact that the experimental fish were not always in optimal spawning condition in all the studies. At least one lab found subtle, but consistent, alterations in plasma VTG and steroid concentrations in fish exposed to flutamide (Jensen et al 2004; Jensen and Ankley 2006). Importantly, flutamide,

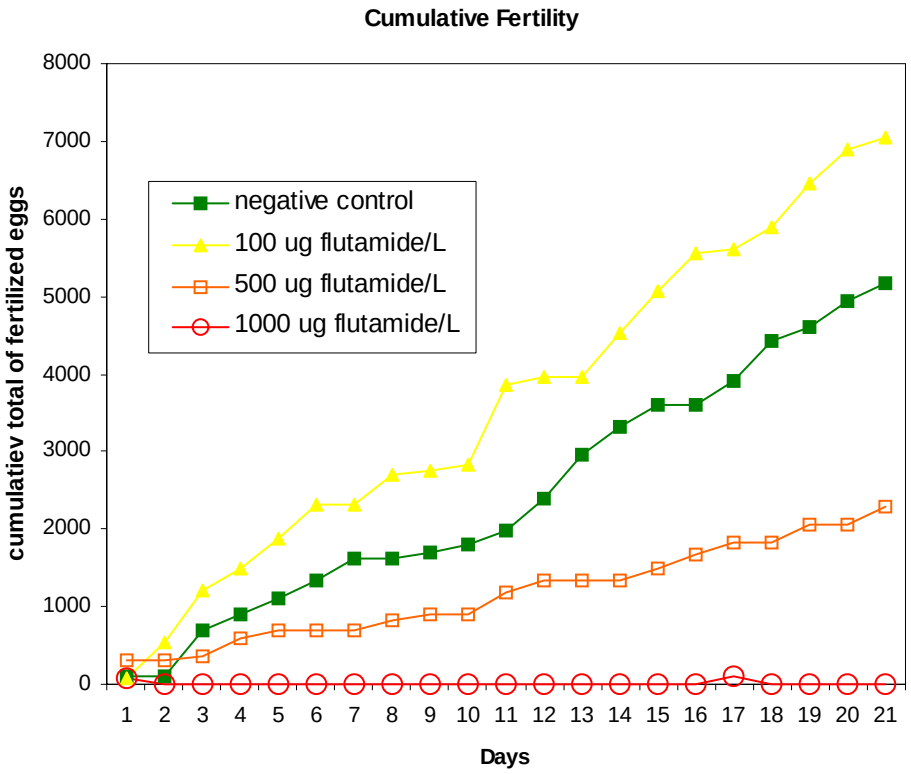
in a dual treatment version of the assay, was demonstrated to block the nuptial tubercle induction of 17β-trenbolone co-treated female fathead minnows, thus confirming the chemical as an androgen receptor antagonist (Ankley et al 2004).

Figure 5. Plot of Cumulative Fecundity by Treatment for the 21-Day Flutamide Assay



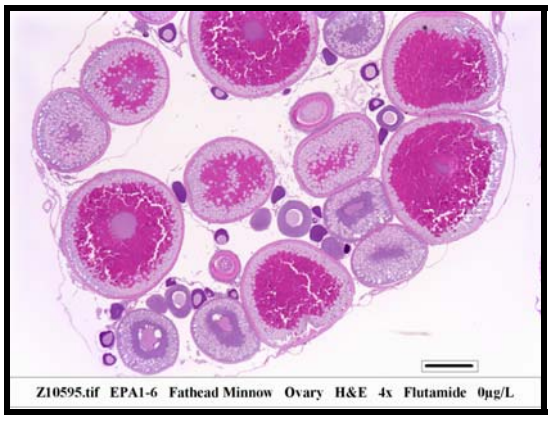
[Battelle 2006](Treatment groups diverge at day 0 with the medium and high treatment groups diverging from the control in a dose responsive pattern. The fecundity of the low treatment group is greater than control.)

Figure 6. Plot of Cumulative Fertility by Treatment for the 21-Day Flutamide Assay

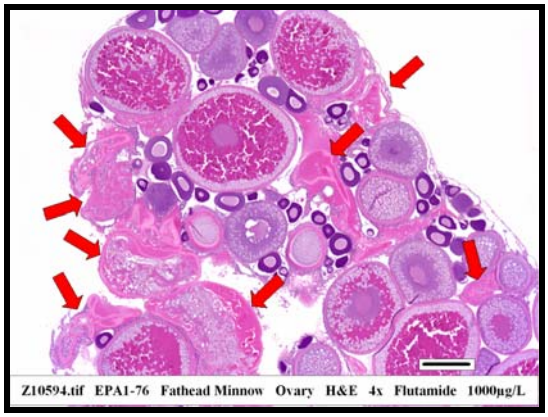


[Battelle 2006](Treatment groups diverge at day 0 with the medium and high treatment groups diverging from the control in a dose responsive pattern. The fertility of the low treatment group is greater than control.)

Figure 7. Increased oocyte atresia observed in flutamide treated fish.

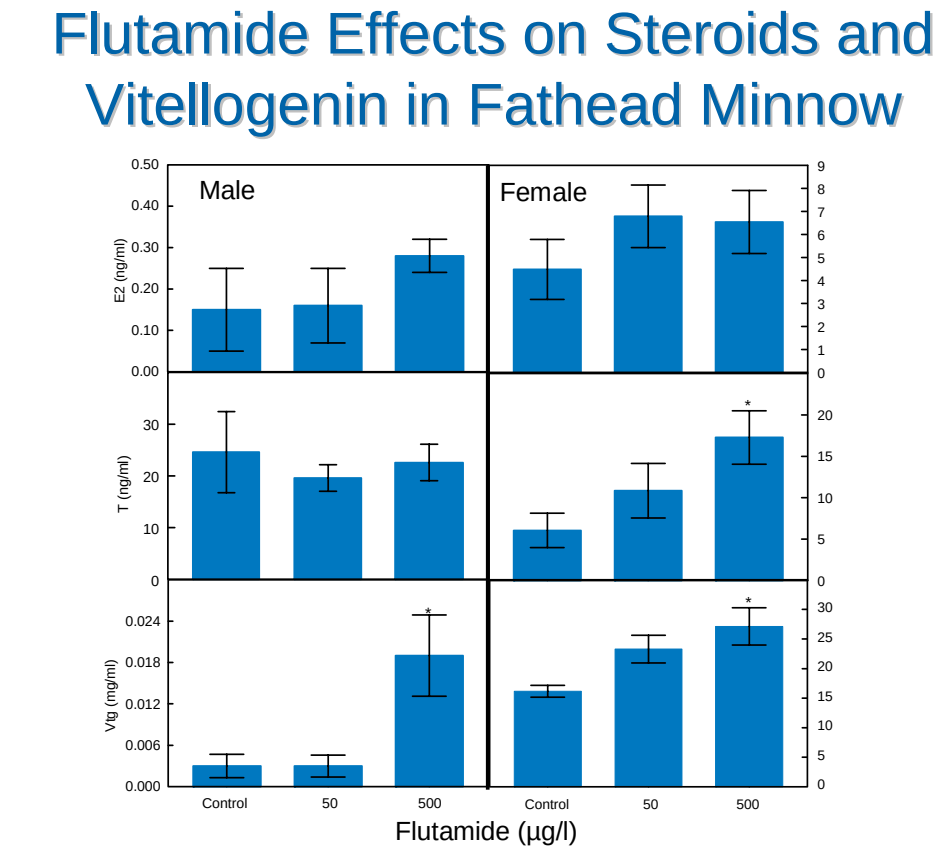


Ovary from a control group female. H&E, bar = 250 microns.



Ovary from a 1000 µg/L flutamide group female. Numerous atretic oocytes are evident (arrows). H&E, bar = 250 microns.

Figure 8.



Jensen et al. (2004) Aquat. Toxicol.

Two other androgen receptor antagonists have been tested using the basic assay described in Addendum 2. For the weak mammalian androgen antagonist p,p'-DDE, histopathology was the only assay endpoint to exhibit a significant response (Table 7). In the case of vinclozolin, another established mammalian androgen receptor antagonist, histopathology and fecundity in the fathead minnow were both responsive (Table 8).

Table 7. p,p'-DDE

Endpoints	(Battelle 2004)
Histopathology	
Male	-
Female	
increased oocyte atresia	+
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	-
Female	-
Fecundity	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 8. Vinclozolin

Endpoints	(Makynen et al 2000), (Ankley et al unpublished data)
Histopathology	
Male	-
Female	
altered gonadal stage (ovary)	+
Vitellogenin	
Male	NR
Female	NR
Secondary sex characteristics	
Male	NR
Female	NR
Fecundity	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Induction of vitellogenin in male fish is well established as a response to estrogen receptor agonists (Brodeur et al 2005; Hansen et al 1998; Harries et al 2000; Korte et al 2000; Kramer et al 1998; Lattier et al 2002; Panter et al 1998; Parks et al 1999; Schmid et al 2002). This was confirmed with the estrogen receptor agonists used in the various 21-day fathead minnow studies summarized in Tables 9 -12.

Effects of 17β-estradiol—a strong estrogen—were consistently detected for nearly every endpoint (Table 9). The only exceptions were in the Phase 1B trials for 17β-estradiol, in which effects on SSC were not detected by one lab, and alterations in fecundity were not observed by two labs. Again, however, the Phase 1B experimental design spawning conditions were suboptimal, which could account for the seeming inconsistency.

For two more weakly estrogenic chemicals that have been tested, Bisphenol A and 4-*tert*-pentylphenol, the assay also consistently detected effects for each endpoint, an exception being for SSC in one study with Bisphenol A (Table 11). One example of findings include reduced or absent nuptial tubercles in males, similar to those reported for a study with 17β-estradiol by Miles-Richardson et al (1999a) (Figure 9). An example of increased proportion of

spermatogonia found in a study with 4-*tert*-pentylphenol is given in Figure 10 (OECD 2006).

Studies with the organochlorine pesticide methoxychlor that used fish in an optimized spawning condition (Table 12) also detected induction of VTG in male fathead minnows, changes in plasma sex steroid concentrations, histopathological alterations, and reductions in fecundity. Estrogenicity is one of several modes of action for methoxychlor, yet these estrogenic effects were the primary findings.

The fact that 17β-estradiol, a strong estrogen used as a positive control, was not always detected by changes in SSC or fecundity demonstrates the desirability of a suite of endpoints that mutually bolster findings. This need is further evident in instances in which problems arise with one or more evaluation. Lab 11 (OECD Phase 1B, Lab 9), for example, experienced difficulty in measuring VTG concentrations, and because neither SSC nor fecundity detected any effects, only histopathological evaluations confirmed effects of this positive control.

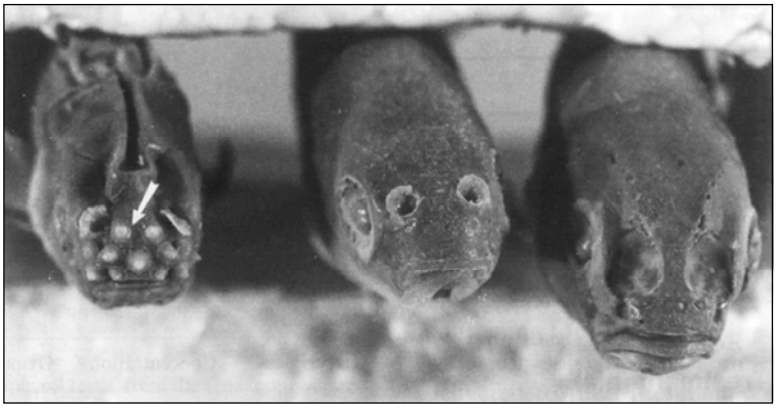
Table 9. 17β-estradiol

Endpoints	(OECD 2006) Lab 9	(OECD 2006) Lab 10	(OECD 2006) Lab 11
Histopathology			
Male			
increased proportion of spermatogonia	+	+	+
additional diagnoses	+	+	+
Female	-	-	+
Vitellogenin			
Male	+	+	NR
Female	+	+	NR
Secondary sex characteristics			
Male	+	+	-
Female	-	-	-
Fecundity	-	+	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Figure 9.

E2 Effects on Male Tubercles



control male	control female	treated male

Miles-Richardson et al. (1999) Aquat. Toxicol.

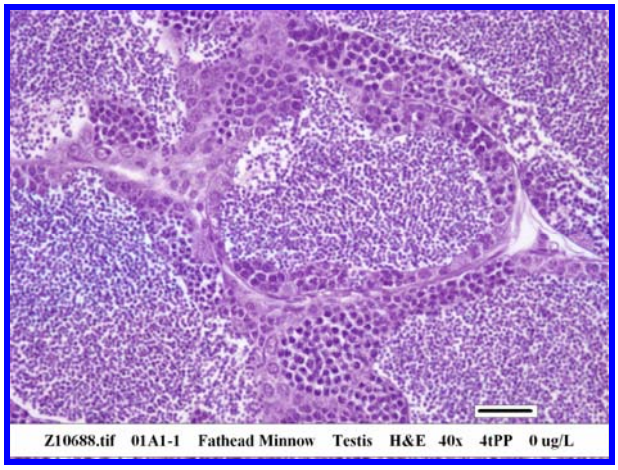
Altered expression of secondary sex characteristics in male fathead minnows: Nuptial tubercles are present in control males, but absent in males treated with 17β-estradiol, similar to untreated females (Miles-Richardson et al 1999a).

Table 10. 4-*tert*-pentylphenol

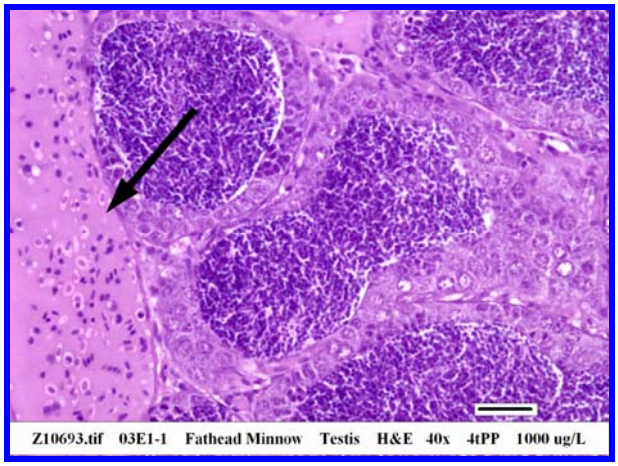
Endpoints	(OECD 2006) Lab 9	(OECD 2006) Lab 10	(OECD 2006) Lab 11
Histopathology			
Male			
increased proportion of spermatogonia	+	+	+
additional diagnoses	+	+	+
Female			
altered gonadal stage	+	+	-
additional diagnoses	-	+	-
Vitellogenin			
Male	+	+	NR
Female	+	-	NR
Secondary sex characteristics			
Male	+	+	+
Female	+	-	-
Fecundity	+	+	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Figure 10. Example of increased proportion of spermatogonia in 4-*tert*-pentylphenol treated male fathead minnows.



Testis from a control male FHM. H&E, bar = 25 microns.



Testis from a male FHM exposed to 1000 µg/L 4tPP. Spermatogonia are the dominant cell type within the germinal epithelium. A large vein contains abundant proteinaceous fluid (plasma) (arrow). H&E, bar = 25 microns.

Table 11. Bisphenol A

Endpoints	(Battelle 2004)
Histopathology	
Male	-
Female	
granulomatous inflammation	+
Vitellogenin	
Male	+
Female	+
Secondary sex characteristics	
Male	-
Female	-
Fecundity	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 12. Methoxychlor

Endpoints	(Ankley et al 2001)	(Battelle 2003a) (21d)	(Battelle 2003a) (14d)
Histopathology			
Male	-	-	-
Female			
increased oocyte atresia	+	-	-
additional diagnoses	-	+	-
Vitellogenin			
Male	+	?	?
Female	-	-	+
Secondary sex characteristics			
Male	-	-	-
Female	-	-	-
Fecundity	+	+	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
? = statistical analysis should be reviewed, apparent large difference between control and high dose group

Steroid synthesis modulators can cause several responses in the 21-day assay through disruption of normal androgen and estrogen production, including alterations in plasma steroid levels, suppression of vitellogenesis, decreased post-ovulatory follicles, impaired maturation of oocytes and spermatozoa, pronounced oocyte atresia, and reduced fecundity. The fish screening assay captures all these responses with the three relevant core endpoints histopathology, VTG, and fecundity (Tables 13 – 16, Figures 10 – 11). Secondary sex characteristics appear to be unresponsive to this mechanism. The chemicals used to investigate the 21-day spawning fish assay's ability to detect steroid synthesis modulators include fadrozole, fenarimol, prochloraz, ketoconazole and cadmium chloride. Abbreviated results for these chemicals are presented in Tables 13 – 17.

Table 13. Fadrozole

Endpoints	(Ankley et al 2002)	(Battelle 2003a) (21d)	(Battelle 2003a)(14d)	(OECD 2006) Lab 10	(OECD 2006) Lab 11	(OECD 2006) Lab 12	(OECD 2006) Lab 13
Histopathology							
Male							
interstitial cell hyperplasia	+	+	+	-	+	+	+
Female							
increased oocyte atresia	+	+	+	+	+	+	+
altered gonadal stage	+	+	+	+	+	+	+
Vitellogenin							
Male	-	-	-	-	NR	-	-
Female	+	+	+	+	NR	-	+
Secondary sex characteristics							
Male	-	-	-	-	-	-	-
Female	-	-	-	-	-	-	-
Fecundity	+	+	+	-	NR	-	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 14. Fenarimol

Endpoints	(Ankley et al 2005a)
Histopathology	
Male	
increased proportion of spermatagonia	+
Female	
increase oocyte atresia	+
Vitellogenin	
Male	-
Female	+
Secondary sex characteristics	
Male	-
Female	-
Fecundity	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 15. Prochloraz

Endpoints	(Ankley et al 2005a)	(OECD 2006) Lab 10	(OECD 2006) Lab 11	(OECD 2006) Lab 12	(OECD 2006) Lab 13
Histopathology					
Male					
additional diagnoses	+	+	-	-	+
Female					
increased oocyte atresia	+	+	+	+	+
additional diagnoses	+	+	-	-	-
Vitellogenin					
Male	-	-	-	-	-
Female	+	+	NR	+	+
Secondary sex characteristics					
Male	-	-	-	-	-
Female	-	-	-	-	-
Fecundity	+	-	+	-	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 16. Ketoconazole

Endpoints	(Ankley and Villeneuve 2006)	(Battelle 2006)
Histopathology		
Male		
interstitial cell hyperplasia	+	+
Female	-	-
Vitellogenin		
Male	-	+
Female	NR	-
Secondary sex characteristics		
Male	NR	-
Female	NR	-
Fecundity	+	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

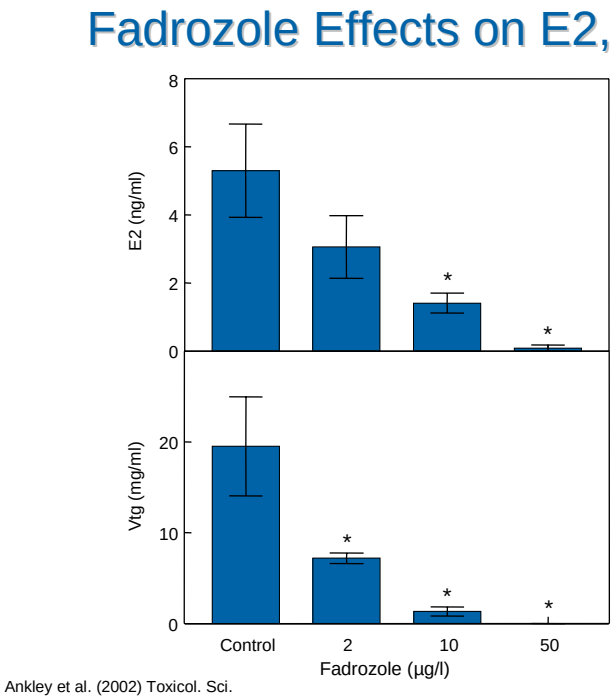
Table 17. Cadmium Chloride

Endpoints	(Battelle 2004)
Histopathology	
Male	-
Female	-
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	-
Female	-
Fecundity	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

The VTG endpoint consistently was affected for fadrozole, prochloraz, and fenarimol in that female vitellogenesis was inhibited. For fadrozole (Table 13), female vitellogenesis was reported as significantly impaired in five out of six laboratories, and at a dose level as low at 2 µg/L (nominal) (Ankley et al 2002) (Figure 11). The reduced VTG was consistent with decreased plasma concentrations of 17β-estradiol, which is consistent with inhibition of aromatase (Figure 11). Prochloraz (Table 15) suppressed female vitellogenesis in four out of four laboratories at dose levels between 100 and 300 µg/L (actual), and fenarimol (Table 14) also significantly suppressed female vitellogenesis (Ankley et al 2005a). Male vitellogenesis was not significantly affected for any of the three compounds. In contrast, with ketoconazole (Table 16), female vitellogenesis was unaffected, whereas male vitellogenesis induction was found in one out of two laboratories. This result was unanticipated because ketoconazole purportedly depresses testosterone synthesis, and therefore should seemingly suppress the production of both estrogens and androgens. The vitellogenesis endpoint was not affected by cadmium chloride (Table 17) exposure.

Figure 11.



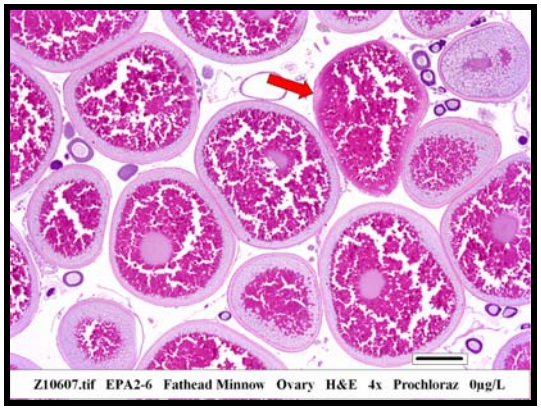
Ankley et al. (2002) Toxicol. Sci.

Histopathology consistently detected effects of all of the steroidogenesis inhibitors except cadmium chloride. For fadrozole, prochloraz, and fenarimol, the most consistent diagnostic criterion was increased oocyte atresia. Seven out of seven labs reported a significant increase in oocyte atresia for fadrozole (see Figure 12 as an example), and five of five laboratories reported increased oocyte atresia for prochloraz. Altered gonadal stage (ovaries) was also reported in seven out of seven laboratories that tested fadrozole. An additional significant histopathological lesion reported for fadrozole, was decreased deposition of VTG (Figure 12, 13), a finding consistent with female vitellogenesis suppression. The testes of the fathead minnow were significantly affected by both fenarimol and ketoconazole. With fenarimol, a significant increase in spermatogonia was detected, potentially as a result of suppressed levels of testosterone leading to alteration of the ability for the spermatogenic cells to mature. With ketoconazole, both laboratories reported interstitial cell hyperplasia (Figure 14), again probably due to suppressed levels of testosterone.

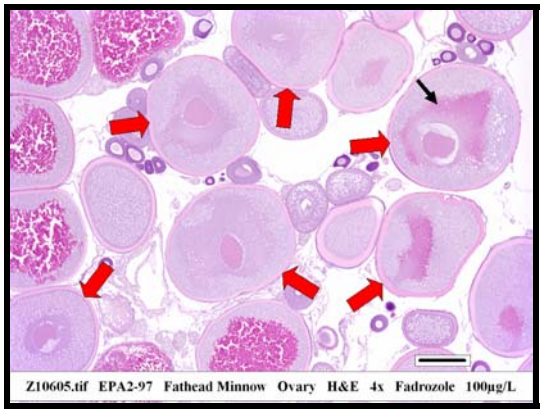
Figure 12. Example of increased oocyte atresia from exposure to fadrozole

Ankley et al. (2002) Toxicol. Sci.

Figure 13. Example of decreased vitellogenin deposition in fadrozole treated fish.



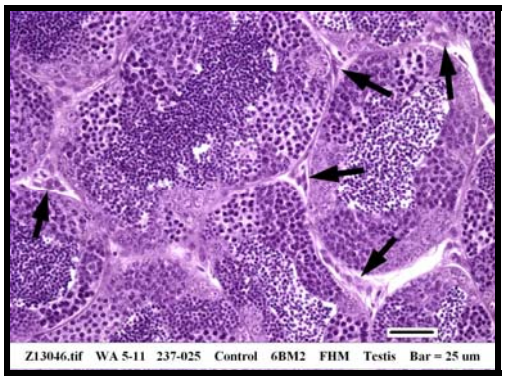
Ovary (Stage 3) from a control group female. A single atretic ovary is evident (arrow). H&E, bar = 250 microns.



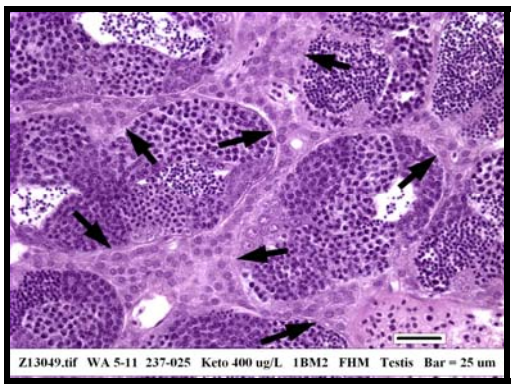
Ovary from a 100 µg/L fadrozole group female. Fadrozole was used as a positive control in the prochloraz study. Decreased vitellogenesis is characterized by the presence of oocytes in which yolk material is not present despite their relatively large size (large arrows). Note that oocytes are affected to varying degrees. Some affected oocytes have extremely fine vitellogenic granules (small arrow), and this is interpreted as ineffective vitellogenesis. H&E, bar = 250 microns.

(OECD 2006)

Figure 14. Example of Interstitial cell hyperplasia in ketoconazole treated fish.



Testis from a control group male. Interstitial areas contain small aggregates of interstitial (Leydig) cells (arrows). Most interstitial cells have wispy, pale cytoplasm. H&E.



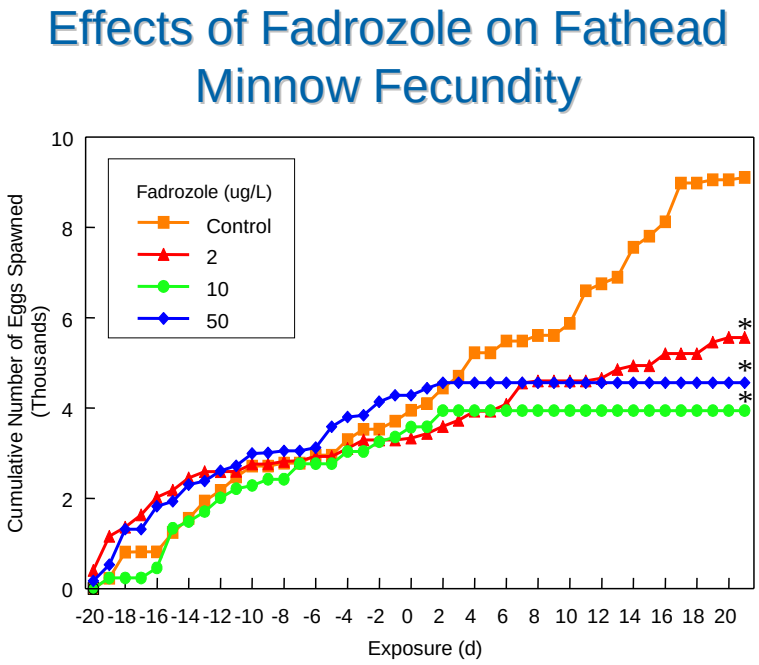
Testis from a 400 µg/L ketoconazole group male. Interstitial cell aggregates (arrows) in the testis of this fish are larger than in control fish, and the cells tend to fill and expand the interstitial spaces. This was diagnosed as Increased Cells, Interstitial Cells, Grade 2 (mild) severity. H&E.

(Battelle Memorial Institute, *unpub.*)

Fecundity was significantly affected (decreased) by exposure to fadrozole (three out of six labs, Figures 16A and B), fenarimol, prochloraz (two out of five labs, Figure 17), and ketoconazole (one out of two labs). Again, much of the fecundity data (for fadrozole and prochloraz) are derived from the OECD Phase 1B study which due to suboptimal spawning design, renders interpretation of fecundity data somewhat problematic. As expected, secondary sex characteristics were not significantly affected by exposure to any steroidogenesis inhibitors. Cadmium chloride did not affect either endpoint in the fish.

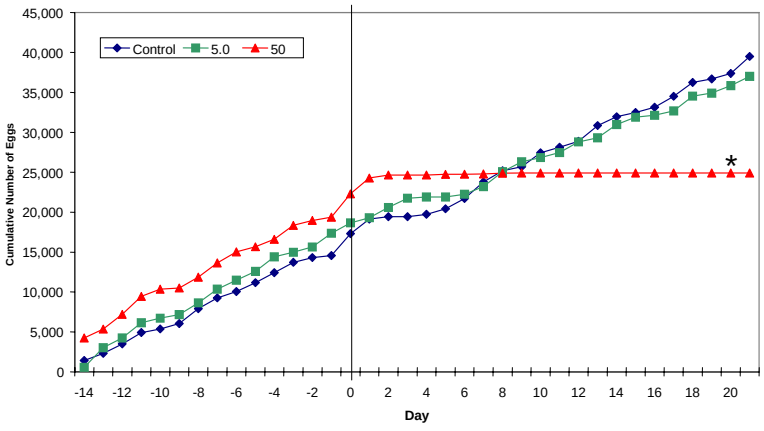
Figure 15.

A.



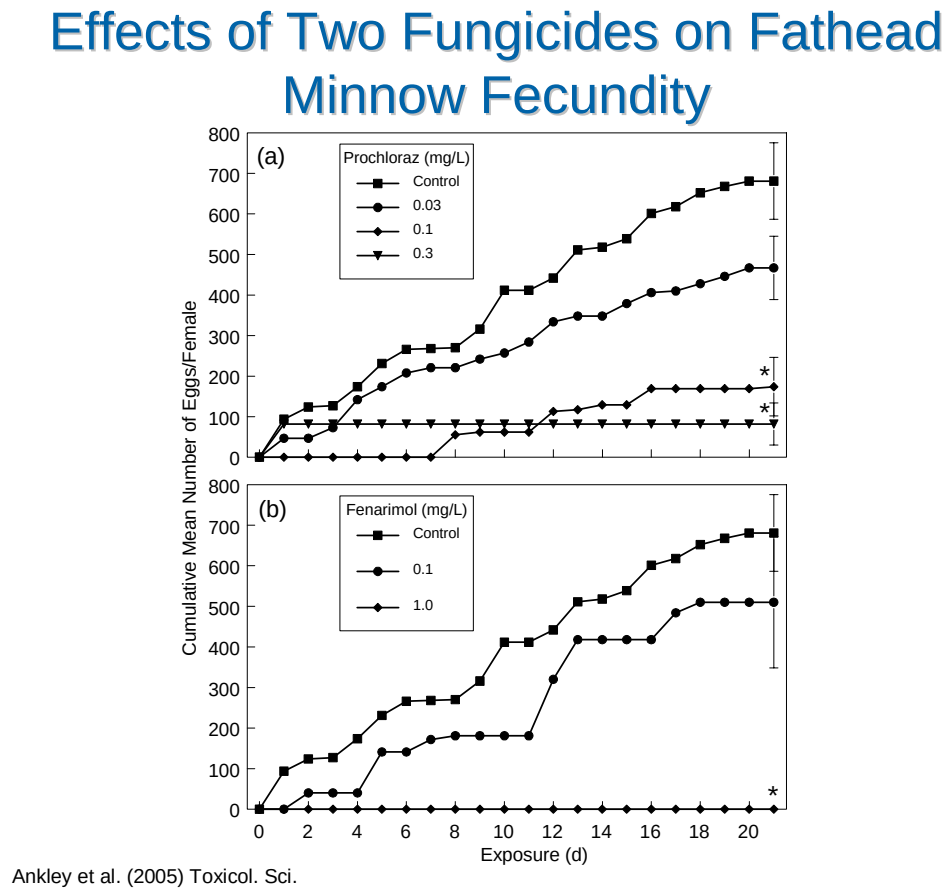
Ankley et al. (2002) Toxicol. Sci.

B.



Battelle (2003)

Figure 16. Effects of prochloraz and fenarimol on fecundity



Ankley et al. (2005) Toxicol. Sci.

Overall, the endpoint that most consistently detected (four out of five) purported steroid synthesis modulators was histopathology. Vitellogenin (decreases in females) consistently detected three of the steroid synthesis modulators (fadrozole, fenarimol and prochloraz), and four of five chemicals affected fecundity, albeit in a less consistent inter-laboratory manner. Only cadmium chloride failed to produce responses in the 21-day assay.

Two studies have used atrazine as a test chemical in the 21-day fathead minnow assay, with one laboratory finding a significant effect (Table 18). It might be anticipated that the fish's reproductive system would be affected by atrazine, as it is considered to be active at the neuroendocrine level (hypothalamus-pituitary axis) in mammals (Cooper et al 1999). Although male fathead minnows showed an increased proportion of spermatogonia following exposure to atrazine in one study, this was not accompanied by changes in fecundity, VTG, or SSC (Table 18).

Table 18. Atrazine

Endpoints	(Battelle 2004)	(Bringolf et al 2004)
Histopathology		
Male		
increased proportion of spermatogonia	+	-
additional diagnoses	+	-
Female	-	-
Vitellogenin		
Male	-	-
Female	-	-
Secondary sex characteristics		
Male	-	-
Female	-	-
Fecundity	-	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Villeneuve et al. (2006) found that the related triazine herbicide prometon caused significant effects on histopathology as seen in increased oocyte atresia as well as decreased post-ovulatory follicles (Table 19). The chemical also caused significant changes to SSC in male fathead minnows. Male fat pad weights relative to body weights were significantly reduced, which might be indicative of anti-androgenic activity. However, there was no clear mechanism of action, as no response was observed in plasma VTG or sex steroid concentrations, fecundity or fertility measurements, aromatase activity, or tubercle index.

Table 19. Prometon

Endpoints	(Villeneuve et al 2006)
Histopathology	
Male	-
Female	
increased oocyte atresia	+
additional diagnoses	+
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	+
Female	-
Fecundity	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Ankley et al. (2005b) speculated that PFOS might affect reproduction in fish through alterations to the HPG axis. Chemicals structurally similar to PFOS can alter plasma concentrations of sex steroids in fathead minnow (Oakes et al 2004). Endpoints that have traditionally been sensitive to alterations to the HPG axis in the fathead minnow include measurements of fecundity and plasma levels of sex steroids and vitellogenin (Ankley et al 2005b). As demonstrated in this study, exposure to PFOS reduced cumulative fecundity in the fathead minnow and caused increased oocyte atresia, altered gonadal stage, and decreased post-ovulatory follicles in females (Table 20). Histopathological changes in females were consistent with the failure to develop and release eggs. Fecundity and histopathological criteria were shown to be sensitive endpoints for this chemical, demonstrating reproductive effects. Although Ankley et al. (2005b) found some evidence of altered plasma steroid concentrations in exposed fish, it is uncertain whether or not these effects were modulated through the HPG axis.

Table 20. PFOS

Endpoints	(Ankley et al 2005b)
Histopathology	
Male	-
Female	
increased oocyte atresia	+
additional diagnoses	+
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	-
Female	-
Fecundity	+

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Potassium permanganate and perchlorate were selected as a toxic negative compound to evaluate how general stress responses would affect estrogen and androgen mediated processes (Tables 21 and 22). The results of the tests indicate that when exposures exceed lethal thresholds, these processes are indeed disturbed. With the exception of a positive histology response (oocyte atresia) for perchlorate, exposure concentrations below lethal limits did not significantly affect the four core endpoints.

Table 21. Perchlorate

Endpoints	(Battelle 2004)
Histopathology	
Male	-
Female	
increased oocyte atresia	+
Vitellogenin	
Male	-
Female	-
Secondary sex characteristics	
Male	-
Female	-
Fecundity	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
NR = not reported, endpoint not included in study

Table 22. Potassium permanganate

Endpoints	(Battelle 2006) Lab 15	(Battelle 2006) Lab 17
Histopathology		
Male		
increased proportion of spermatogonia	+(m)	+
additional diagnoses	+(m)	-
Female		
altered gonadal stage	+(m)	-
Vitellogenin		
Male	-	-
Female	+(m)	-
Secondary sex characteristics		
Male	+(m)	-
Female	-	-
Fecundity	+(m)	-

+ = significant or pathologist observed change from control
- = no significant or pathologist observed change from control
m = observed only at concentrations exhibiting mortality
NR = not reported, endpoint not included in study

Test method reliability

The fish short-term reproduction assay with the combination of endpoints has been demonstrated by efforts in the U.S. to be capable of detecting known endocrine-active compounds. In the OECD Phase 1A, a non-spawning protocol was used which did not include a fecundity endpoint, but did include the histopathology endpoint. In fact, the histopathology identified disturbances in the gonads of control fishes that were determined to be related to the non-spawning status, and therefore led to changes in Phase 1B to allow spawning (Lothenbach et al 2005). This supports the idea that histopathology is useful to determine the general health of the fish, and acts as an internal control for the experiment. However, the VTG endpoint in the Phase 1A did respond to the potent estrogen agonist (17β-estradiol) and the potent androgen agonist (17β-trenbolone) and SSC in female medaka and fathead minnow were similarly responsive to 17β-trenbolone. In the OECD Phase 1B, the VTG endpoint responded consistently to both the weak estrogen and steroidogenesis inhibitor. The VTG endpoint performed poorly for the anti-androgen (flutamide), and consistent responses in SSC were not observed for any of the test compounds.

The spawning and histopathology endpoints in Phase 1B provided mixed results, which were likely due to the inconsistencies in spawning observations (qualitative or quantitative), spawning configuration for fathead minnows, maturation/spawning condition of the fish, and inconsistent reporting for histopathology. Nonetheless, spawning and histopathology were generally the most sensitive endpoints (or at least comparable to VTG). For flutamide (an anti-androgen), the only two endpoints that identified activity for this compound in Phase 1B were spawning in the fathead minnow and histopathology in zebrafish.

The variability for egg production measures in the fathead minnow assay, as performed in the U.S. trials, is often less than what is observed for plasma VTG measures (Table 23). In considering the variability associated with histopathology, it must be understood that the diagnostic criteria or specific lesions do not represent separate discreet endpoints and should not be evaluated in this context. It is the summary pathology reading and overall response of the organ that represents the endpoint. In this regard, the pathology experts at the Phase 1B histopathology consultation "…concluded that overall exposure-related findings were generally consistent among different laboratories" in the Phase 1B trials (OECD 2004c).

Table 23.

Mean values and variability of fecundity & VTG endpoints in control females

Control	Fecundity			Vitellogenin		
Treatment	Eggs/♀/day			(mg/mL)		
Group	Mean	SD[a]	CV[b]	Mean	SD	CV
Atrazine	53.6	13.1	24%	6.6	2.4	36%
Bisphenol A	57.8	10.6	18%	11.1	3.4	31%
Cadmium	63.9	18.3	29%	10.7	2.5	23%
p,p-DDE	65.5	9.1	14%	11.5	2.6	22%
Perchlorate	61.3	8.3	14%	8.3	2.0	24%
Study Mean	60.4	11.8	20%	9.6	3.1	32%

Battelle (2004)

Data interpretation

The U.S. endocrine screening and testing framework intends to use a weight-of-evidence (WOE) approach for data interpretation, which includes WOE of the endpoints within an assay (with some endpoints more important than others) and WOE of findings from a battery of assays (*in vitro* and *in vivo*). It is the battery assessment which dictates the conclusions relevant to endocrine disruption and triggers for further testing. However, an apical endpoint such as fecundity has relevance beyond EDCs. Impaired fecundity is an adverse effect with regulatory importance whether it is due to endocrine-mediated activity or another MOA. Evaluation of a possible fecundity effect would have to consider the relative concentration which elicits the effect. If fecundity alone is affected and it is only affected at concentrations which also cause mortality, weight loss, or other obvious sign of toxicity, then it would likely be discounted. If it occurs at sublethal (toxic) levels, on the other hand, then a dose-response would likely be sought with additional higher tier testing.

A suite of endpoints (VTG, SSC, histopathology, and fecundity) are needed (in the U.S. opinion) to allow a conclusion of "no effect" in the fish short-term reproduction assay. A "no effect" conclusion would pass a compound through the screen and relieve it from further consideration. The endpoints together buttress each other for WOE review. The utility of the assay extends beyond potential endocrine-active substances to also include possible fish reproductive toxicants and meets regulatory needs currently unfulfilled in both cases.

Assay validation status

It should be clear from the information presented that there are ample data available which can support the reliability of a fish screening assay that includes a comprehensive suite of endpoints to detect compounds known to interfere with endocrine-mediated processes or otherwise affect reproduction. Likewise, the available information demonstrates the inability of the assay to detect all known ED substances when limited to just VTG and SSC endpoints. Even though no single endpoint has proven to be the most sensitive and most consistently responsive for all known estrogen and androgen mediated process-disrupting agents, the suite of endpoints has been shown to effectively capture and inform the need for further testing on these agents. It is also recognized that the assay is able to serve broader hazard identification purposes. Although these data are only briefly and generally summarized in this paper, it should be apparent that there is a solid basis to develop an integrated validation summary report to advance to a peer review on validation status for the intended purpose of the assay as it is defined in this paper. It remains a prerogative of a peer

review panel to agree or disagree with the validity of the assay and the suite of endpoints as recommended.

In considering what conclusions may be drawn from the available data, the following points are provided:

(1) Androgens cause clear, unambiguous changes in secondary sex characteristics in females in the assay and trigger further testing. The fact that the other endpoints are altered, vitellogenin, fecundity, histology (and steroids) is added information, in terms of the screen's ability to flag a compound, *only* to better understand toxicity pathways (VTG, steroids, histology) or reproductive impacts (fecundity).

(2) Estrogens cause a clear, unambiguous increase in VTG in males. The fact that the other endpoints can be altered, secondary sex characteristics, fecundity, histology (and steroids) is added information *only* to better understand toxicity pathways (steroids, histology) or reproductive impacts (fecundity).

(3) Several chemicals known as inhibitors of different steps in steroidogenesis (fadrozole, prochloraz, fenarimol) decrease VTG in females, although the *in vivo* steroid data are somewhat ambiguous that this is by inhibition of steroidogenesis. Again, the fact that other endpoints can be altered is important only to better understand toxicity pathways (*e.g.*, we can identify which enzymes might be inhibited by looking at steroids) or specific reproductive impacts.

The exception here-and it may be an important one- is ketoconazole, which we know from mammalian studies *should be* an ED compound. The fish assay is positive for fecundity effects indicating that it is a reproductive toxicant. It also is positive for gonad histology effects that are specific enough (interstitial cell hyperplasia) to suggest an endocrine pathway etiology.

(4) Based on all the data in the analysis, a strong case cannot be made that androgen receptor antagonists are effectively identified by the assay as "endocrine active". The exception may be the US-EPA MED laboratory which includes serum steroid analysis. In considering flutamide, there seems to be a consistent effect on fecundity, which means that it would be flagged as a reproductive toxicant. However, neither secondary sex characteristics nor vitellogenin would identify it as a potential ED compound, and the primary histological effects (oocyte atresia) are too general to do so. Oocyte atresia can be caused by many things in addition

to ED, including flawed experimental designs-see OECD Phase 1A. The vinclozolin fecundity and histology data that are presented, although more limited than flutamide, offer exactly the same picture-that of a reproductive toxicant, not an ED substance. And, the p,p-DDE data show only the general histopathology finding (oocyte atresia) and no fecundity response.

Overall, if you consider all the data, one may conclude that the assay-with all four endpoints-will detect anti-androgenic chemicals, as at least reproductive toxicants.

(5) Next, it is necessary to consider what is summarized for the three chemicals—atrazine, PFOS and prometon—with undefined "endocrine" modes of action. It should be understood that the primary mode of action for the three compounds may not in fact be endocrine-mediated, and their endocrine modalities only secondary to other action. The results for these compounds were somewhat mixed in demonstrating the assay's effectiveness. Two of the chemicals-atrazine and prometon-were not identified as reproductive toxicants (to the extent that fecundity was reduced). Both triazines caused oocyte atresia, a relatively non-specific histological response. PFOS both reduced fecundity and caused oocyte atresia, so it can be construed to be a reproductive toxicant. The only specific endocrine-mediated response from these three studies was a decrease in male fatpad weight in prometon-exposed males. However, there is limited experience with this measurement that has only recently been included in the secondary sex characteristics endpoint.

So, where does that leave the "unknown" analysis? There are three chemicals with undefined modes of action that all cause oocyte atresia-the same response as seen for anti-androgens and a negative control (perchlorate)-and one chemical (PFOS) that also decreased fecundity.

(6) Finally, we need to consider the two chemicals identified as "toxic negatives". Potassium permanganate in one lab shows that all the endpoints respond when lethal thresholds are exceeded. In another lab, histology (in males), but not fecundity, is affected. Perchlorate also caused effects on (female) histology but not fecundity. However, when concentrations are below overt toxicity (lethal) levels, the endpoints do not spuriously respond.

The fish short-term reproduction screening assay, as proposed, can be considered to address two roles – to detect potential endocrine disrupting agents or to detect reproductive toxins. There is no debate that changes in VTG and SSC

are indicative of endocrine-disrupting effects. There should also be no debate that fecundity (and fertility, etc.) and histopathology are appropriate for identifying potential reproductive toxicants. The debate that exists is on the split purpose of the assay. The U.S. accepts both purposes and contends that these are not altogether mutually exclusive. Substances that interfere with the normal function of androgens and estrogens also affect reproduction. Additionally, reproductive toxicants which act through non-hormonally mediated action are also of potential regulatory concern. The only difference between a fish screening protocol specific to ED and one targeting reproductive toxins, are the observational endpoints included. The in-life conduct and numbers of fish used are the same. It is irresponsible to not collect and evaluate the fecundity data provided by spawning fish and collect the reproductive organ for assessing possible pathology at assay termination in addition to vitellogenin and secondary sex characteristics endpoints in a single assay especially where it would save the use of animals in a separate, virtually identical assay.

In addition to detecting an ED and/or reproductive effect and triggering higher tier testing, the screening assay should be able to inform the appropriate concentration range to be used in a definitive test. This would avoid the need for an additional range-finding study and further reduce the number of animals needed overall in hazard assessment. A screening assay limited to VTG and SSC would be inadequate in this matter, where information included for fecundity and histopathology would be invaluable.

The standardized protocol presented in Addendum 2 has not received a formal interlaboratory evaluation. Although the general design and components are by and large identical to many of the studies summarized in this discussion paper which support the assay reliability, there exist some elements which have been modified to improve the test guidance and practicality. For this reason, it is appropriate to evaluate the standardized protocol in a limited interlaboratory exercise. The US-EPA proposes to perform this interlaboratory trial with a minimum of 3 laboratories and 2 test chemicals using the fathead minnow. It is recommended that one of the chemicals will be well demonstrated to be consistently responsive across the four core endpoints in the assay, and the second chemical will be one with limited experience in the fathead minnow assay but expected to be responsive for one or more core endpoints. This exercise is intended to confirm the reproducibility of the assay for its designated purpose as indicated by the prevailing data. Information on the identity of the test chemicals will be limited (e.g., coded for certain endpoint analysis where practical) to avoid any potential bias.

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Addendum 1

Summary results of fathead minnow screening assays

Laboratory & protocol information for studies included

Laboratories

Lab 1	EPA MED-Duluth	
Lab 2	Battelle-SEQUIM	
Lab 3	Iowa State	static-renewal aquatic system as opposed to flow-through
Lab 4	Michigan State	
Lab 5	Astra-Zeneca	
Lab 6	Germany	
Lab 7	Brunel University	
Lab 8	Univ. of Guelph	
Lab 9	Lab 7 of OECD Phase 1B	
Lab 10	Lab 8 of OECD Phase 1B	
Lab 11	Lab 9 of OECD Phase 1B	
Lab 12	Lab 11 of OECD Phase 1B	
Lab 13	Lab 4 of OECD Phase 1B	
Lab 14	Lab 10 of OECD Phase 1B	
Lab 15	ABC labs	
Lab 16	Wildlife International	
Lab 17	Springborn Smithers	

Protocols

	Duration	**Spawning/Non-spawning**	**Female:Male**	
Protocol 1	21 days	spawning	4:2	
Protocol 2	21 days	spawning	1:1	
Protocol 3	14 days	spawning	4:2	
Protocol 4	14 days	spawning	1:1	
Protocol 5	9-12 days	spawning	4:2	
Protocol 6	14 days	non-spawning	sexes kept separate	
Protocol 7	10 days	sexually mature (6-8 months old)	4:2	
Protocol 8	21 days	spawning	3:3	
Protocol 9	21 days	non-spawning	unknown - 7 randomly chosen per tank	only a 24 hour acclimation period was used
Protocol 10	4, 7, 14, 21, 32 days	non-spawning	unknown - 8 randomly chosen per tank	14 day acclimatization period used
Protocol 11	21 days	non-spawning	sexes kept separate	
Protocol 12	21 days	fish kept in gonadal quiescence until start of test	1:1	
Protocol 13	42 days	spawning	1:1	
Protocol 14	28 days	spawning	4:2	
Protocol 15	21 days	spawning	5:5	
Protocol 16	7 days	spawning?	4:2	

Methyltestosterone

	Core Diagnostic Criteria	Lab 1 - protocol 5 (Ankley et al 2001)	Lab 1 - protocol 16 (Hornung et al 2004)	Lab 6 - protocol 12 (Pawlowski et al 2004a)
Males	Testis-ova	-	NR	NR
	Increased proportion of spermatogonia	-	NR	NR
	Interstitial cell hypertrophy/hyperplasia	-	NR	NR
	Increased testicular degeneration	+ (0.2 mg/L[#])	NR	NR
Females	Increased oocyte atresia	+ (0.2 mg/L[#])	NR	+ (0.09 µg/L)
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR
	Decreased yolk formation	NR	NR	NR
	Altered gonadal stage	+ (0.2 mg/L[#])	NR	-
	Secondary Diagnostic Criteria			
Males	Decreased proportion of spermatogonia	+ (0.2 mg/L[#])*	NR	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR
	Asynchronous gonad development	NR	NR	NR
	Altered gonadal staging	+ (0.2 mg/L[#])	NR	NR
Females	Granulomatous inflammation	NR	NR	NR
	Interstitial fibrosis	NR	NR	NR
	Egg debris in the oviduct	NR	NR	NR
	Granulomatous inflammation	NR	NR	NR
	Decreased post-ovulatory follicles	+ (0.2 mg/L[#])	NR	NR
	Vitellogenin			
	Males	+ (0.2 mg/L[#])	+ (20 µg/L[#])	+ (1 µg/L[#])
	Females	+ (0.2 mg/L[#])	+ (20 µg/L[#])	+ (1 µg/L[#])
	Secondary sex characteristics			
	Males	+ (0.2 mg/L[#])	-	-
	Females	+ (0.2 mg/L[#])	+ (20 µg/L[#])	+ (1 µg/L[#])
	Fecundity	+ (↓ 0.2 mg/L[#])	NR	+(↓ 48.2 µg/L)

NR = Not reported

Lab 1 - protocol 2 reported a significant decrease in spermatogenesis in treated males

*Report of hyperproduction of sperm - is this associated with decreased proportion of spermatogonia?

= nominal versus actual concentration

β–trenbolone

	Core Diagnostic Criteria	Lab 1 - protocol 1 (Ankley et al 2003)	Lab 2 - protocol 1 (Battelle 2003a)	Lab 2 - protocol 3 (Battelle 2003a)	Lab 2 - protocol 6 (Battelle 2003a)
Males	Testis-ova	-	-	-	-
	Increased proportion of spermatogonia	-	-	-	-
	Interstitial cell hypertrophy/hyperplasia	-	-	-	-
	Increased testicular degeneration	-	NR	NR	NR
Females	Increased oocyte atresia	+ (5 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	-
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR	NR
	Decreased yolk formation	+ (5 µg/L[#])	NR	NR	NR
	Altered gonadal stage	+ (5 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	+ (0.071 µg/L)
	Secondary Diagnostic Criteria				
Males	Decreased proportion of spermatogonia	+ (5 µg/L[#])*	+ (0.60 µg/L)	-	-
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR	NR
	Asynchronous gonad development	NR	NR	NR	NR
	Altered gonadal staging	+ (5 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	-
Females	Granulomatous inflammation	NR	-	-	-
	Interstitial fibrosis	NR	NR	NR	NR
	Egg debris in the oviduct	NR	NR	NR	NR
	Granulomatous inflammation	NR	-	-	-
	Decreased post-ovulatory follicles	+ (5 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	NR
	Vitellogenin				
	Males	+ (50 µg/L[#])	-	-	+ (↓ 0.071 µg/L)
	Females	+ (↓ 0.05 µg/L[#])	+ (↓ 0.60 µg/L)	+ (↓ 0.78 µg/L)	+ (↓ 0.071 µg/L)
	Secondary sex characteristics				
	Males	-	-	-	-
	Females	+ (0.05 µg/L[#])	+ (0.60 µg/L)	+ (0.78 µg/L)	+ (0.86 µg/L)
	Fecundity	+ (↓ 0.05 µg/L[#])	+ (↓ 0.60 µg/L)	+ (↓ 0.78 µg/L)	NA

Orange header row (Lab 2 – protocol 6) indicates 14-day non-spawning assay
NR = Not reported
Lab 1 - protocol 1 reported a significant increase in testis tubule lumen size with treated males
*Report of hyperproduction of sperm - is this associated with decreased proportion of spermatogonia?
= nominal versus actual concentration
Pink highlighting indicates that there is discrepancy between the published paper and the table

		α-trenbolone (Jensen et al 2006)
	Core Diagnostic Criteria	
Males	Testis-ova	NR
Males	Increased proportion of spermatogonia	NR
Males	Interstitial cell hypertrophy/hyperplasia	NR
Males	Increased testicular degeneration	NR
Females	Increased oocyte atresia	NR
Females	Perifollicular cell hypertrophy/hyperplasia	NR
Females	Decreased yolk formation	NR
Females	Altered gonadal stage	NR
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	NR
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR
Males	Asynchronous gonad development	NR
Males	Altered gonadal staging	NR
Females	Granulomatous inflammation	NR
Females	Interstitial fibrosis	NR
Females	Egg debris in the oviduct	NR
Females	Granulomatous inflammation	NR
Females	Decreased post-ovulatory follicles	NR
	Vitellogenin	
	Males	-
	Females	+ (↓ 0.032 µg/L)
	Secondary sex characteristics	
	Males	-
	Females	+ (0.032 µg/L)
	Fecundity	+ (0.0097 µg/L)

NR = not reported

p,p'-DDE

		Lab 2 - Protocol 1 (Battelle 2004)
	Core Diagnostic Criteria	
Males	Testis-ova	-
Males	Increased proportion of spermatogonia	-
Males	Interstitial cell hypertrophy/hyperplasia	-
Males	Increased testicular degeneration	NR
Females	Increased oocyte atresia	+ (0.022 µg/L)
Females	Perifollicular cell hypertrophy/hyperplasia	NR
Females	Decreased yolk formation	NR
Females	Altered gonadal stage	-
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR
Males	Asynchronous gonad development	NR
Males	Altered gonadal staging	-
Females	Granulomatous inflammation	-
Females	Interstitial fibrosis	NR
Females	Egg debris in the oviduct	NR
Females	Granulomatous inflammation	-
Females	Decreased post-ovulatory follicles	-
	Vitellogenin	
	Males	-
	Females	-
	Secondary sex characteristics	
	Males	-
	Females	-
	Fecundity	-

NR = not reported

Flutamide, part 1 (protocols 1 & 15) – continued on next page

		Phase 1B studies						
		Lab 1 - protocol 1 (Jensen et al 2004)	Lab 2 - protocol 1 (Battelle 2003a)	Lab 9 - protocol 15 (OECD 2006)	Lab 14 - protocol 15 (OECD 2006)	Lab 13 - protocol 15 (OECD 2006)	Lab 16 - protocol 1 (Battelle *2006*)	Lab 17 - protocol 1 (Battelle 2006)
Males	Testis-ova	NR	-	-	-	-	-	-
	Increased proportion of spermatogonia	-	-	-	+ (445 µg/L)	-	+ (388 µg/L)	-
	Interstitial cell hypertrophy/hyperplasia	NR	-	-	-	-	-	-
	Increased testicular degeneration	+ (50 µg/L[#])*	NR	-	-	-	-	-
Females	Increased oocyte atresia	+ (500 µg/L[#])	+ (510 µg/L)	-	-	+ (68.7 µg/L)	+ (822 µg/L)	-
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	-	-	-	-	-
	Decreased yolk formation	NR	NR	-	-	-	-	-
	Altered gonadal stage	+ (500 µg/L[#])	-	-	-	-	-	-
	Secondary Diagnostic Criteria							
Males	Decreased proportion of spermatogonia	-	-	-	-	-	-	-
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	-	-	-	-	-
	Asynchronous gonad development	NR	NR	-	-	-	-	-
	Altered gonadal staging	-	-	-	-	-	+ (388 µg/L)	-
	Granulomatous inflammation	NR	-	-	-	-	-	-
Females	Interstitial fibrosis	NR	NR	-	-	-	-	-
	Egg debris in the oviduct	NR	NR	-	-	-	-	-
	Granulomatous inflammation	NR	-	-	-	-	-	-
	Decreased post-ovulatory follicles	NR	-	-	-	-	-	-
	Vitellogenin							
	Males	+ (500 µg/L[#])	-	-	-	-	-	-
	Females	+ (500 µg/L[#])	-	-	-	-	-	-
	Secondary sex characteristics							
	Males	-	-	+ (↓ 754 µg/L)	-	-	+ (↓ 388 µg/L)	+ (↓ 690 µg/L)
	Females	-	-	-	-	-	-	-
	Fecundity	+ (↓ 500 µg/L[#])	+ (↓ 510 µg/L)	+ (↓ 754 µg/L)	+ (↓ 875 µg/L)	+ (↓ 940 µg/L)	+ (↓ 822 µg/L)	-

Flutamide, part 2 (protocols 3, 6, & 11) – continued from previous page

		Lab 2 - protocol 3 (Battelle 2003a)	Lab 2 - protocol 6 (Battelle 2003a)	Lab 5 - protocol 11 (Panter et al 2004)
Males	Testis-ova	-	-	NR
	Increased proportion of spermatogonia	-	-	NR
	Interstitial cell hypertrophy/hyperplasia	-	-	NR
	Increased testicular degeneration	NR	NR	NR
Females	Increased oocyte atresia	+ (519 µg/L)	-	NR
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR
	Decreased yolk formation	NR	NR	NR
	Altered gonadal stage	-	-	NR
	Secondary Diagnostic Criteria			
Males	Decreased proportion of spermatogonia	-	-	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR
	Asynchronous gonad development	NR	NR	NR
	Altered gonadal staging	-	-	NR
	Granulomatous inflammation	-	-	NR
Females	Interstitial fibrosis	NR	NR	NR
	Egg debris in the oviduct	NR	NR	NR
	Granulomatous inflammation	-	-	NR
	Decreased post-ovulatory follicles	+ (519 µg/L)	-	NR
	Vitellogenin			
	Males	-	-	-
	Females	-	+ (260 µg/L)	+**
	Secondary sex characteristics			
	Males	-	-	+ (938.6 µg/L)
	Females	-	-	-
	Fecundity	+ (↓ 519 µg/L)	NA	NA

Orange headed indicates 14-day non-spawning assay
NR = Not reported
*Lab 1 - protocol 1 reported the increased presence of cells with pycnotic nuclei located in the tubule epithelium in flutamide-treated males (considered aborted spermatogonia or sperm precursor cells)
**Dilution water control showed a significant increase in female vitellogenin levels in addition to 95.3 µg/L test level
= nominal versus actual concentration
Purple highlighting indicates control groups did not spawn well

Methoxychlor

	Core Diagnostic Criteria	Lab 1 - protocol 1 (Ankley et al 2001)	Lab 2 - protocol 1 (Battelle 2003a)	Lab 2 - protocol 3 (Battelle 2003a)	Lab 2 - protocol 6 (Battelle 2003a)	Lab 5 - protocol 10 (Panter et al 2002)
Males	Testis-ova	-	-	-	-	NR
	Increased proportion of spermatogonia	-	-	-	-	NR
	Interstitial cell hypertrophy/hyperplasia	-	-	-	-	NR
	Increased testicular degeneration	-	NR	NR	NR	NR
Females	Increased oocyte atresia	+ (0.5 µg/L[#])	-	-	-	NR
	Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR	NR	NR
	Decreased yolk formation	NR	NR	NR	NR	NR
	Altered gonadal stage	NR	-	-	-	NR
	Secondary Diagnostic Criteria					
Males	Decreased proportion of spermatogonia	NR	-	-	-	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR	NR	NR
	Asynchronous gonad development	NR	NR	NR	NR	NR
	Altered gonadal staging	NR	-	-	-	NR
	Granulomatous inflammation	NR	-	-	-	NR
Females	Interstitial fibrosis	NR	NR	NR	NR	NR
	Egg debris in the oviduct	NR	NR	NR	NR	NR
	Granulomatous inflammation	NR	-	-	-	NR
	Decreased post-ovulatory follicles	NR	-	*	-	NR
	Vitellogenin					
	Males	+ (5 µg/L[#])	-**	-**	-	+ (0.16 µg/L)***
	Females	-	-	+ (↓ 0.79 µg/L)	-	NR
	Secondary sex characteristics					
	Males	-	-	-	-	NR
	Females	-	-	-	-	NR
	Fecundity	+ (↓ 5 µg/L[#])	+ (↓ 4.05 µg/L)	+ (↓ 3.98 µg/L)	NA	NA

Orange header (Lab 2, protocol 6) indicates 14-day non-spawning assay
NR = Not reported
NA = Not applicable
*Lab 2 - protocol 1 reported a significant difference in females with increased post-ovulatory follicles in high dose groups
** The statistical analysis of this result should be reviewed as it looks as if there is a large difference in vitellogenin concentrations between controls and high dose groups
*** Significance was achieved in after only four days of exposure to the test chemical, however, at 21 days, the dilution water control males also had significant changes in vitellogenin levels
# - nominal versus actual concentration

Vinclozolin

		Lab 1 - protocol 2 (Makynen et al 2000)
	Core Diagnostic Criteria	
Males	Testis-ova	NR
Males	Increased proportion of spermatogonia	-
Males	Interstitial cell hypertrophy/hyperplasia	NR
Males	Increased testicular degeneration	NR
Females	Increased oocyte atresia	-
Females	Perifollicular cell hypertrophy/hyperplasia	NR
Females	Decreased yolk formation	NR
Females	Altered gonadal stage	+ (1200 µg/L[#])
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR
Males	Asynchronous gonad development	NR
Males	Altered gonadal staging	-
Females	Granulomatous inflammation	NR
Females	Interstitial fibrosis	NR
Females	Egg debris in the oviduct	NR
Females	Granulomatous inflammation	NR
Females	Decreased post-ovulatory follicles	NR
	Vitellogenin	
	Males	NR
	Females	NR
	Secondary sex characteristics	
	Males	NR
	Females	NR
	Fecundity	NR

NR = not reported
# = nominal concentration

17β-estradiol

	Core Diagnostic Criteria	Lab 9 - protocol 15 (OECD 2006)	Lab 10 - protocol 15 (OECD 2006)	Lab 11 - protocol 15 (OECD 2006)	Lab 4 - protocol 7 (Miles-Richardson et al 1999a)	Lab 4 - protocol 8 (Kramer et al 1998)	Lab 5 - protocol 9 (Panter et al 1998)
Males	Testis-ova	-	-	-	NR	NR	NR
Males	Increased proportion of spermatogonia	+ (90.6 ng/L)	+ (100 ng/L#)	+ (100 ng/L#)	NR	NR	NR
Males	Interstitial cell hypertrophy/hyperplasia	-	-	-	+ (2 nM#)*	NR	NR
Males	Increased testicular degeneration	-	+ (100 ng/L#)	-	+ (0.5 nM#)	NR	NR
Females	Increased oocyte atresia	-	-	-	+ (2 nM#)**	NR	NR
Females	Perifollicular cell hypertrophy/hyperplasia	-	-	-	NR	NR	NR
Females	Decreased yolk formation	-	-	-	NR	NR	NR
Females	Altered gonadal stage	-	-	+ (100 ng/L#)	+ (0.1 nM#)	NR	NR
	Secondary Diagnostic Criteria						
Males	Decreased proportion of spermatogonia	-	-	-	NR	NR	NR
Males	Increased proteinaceous fluid within testicular vessels or interstitium	-	+ (100 ng/L#)	+ (100 ng/L#)	NR	NR	NR
Males	Asynchronous gonad development	-	-	-	NR	NR	NR
Males	Altered gonadal staging	-	-	+ (100 ng/L#)	NR	NR	NR
Females	Granulomatous inflammation	-	-	-	NR	NR	NR
Females	Interstitial fibrosis	-	-	-	NR	NR	NR
Females	Egg debris in the oviduct	-	-	-	NR	NR	NR
Females	Granulomatous inflammation	+ (90.6 ng/L)	-	-	NR	NR	NR
Females	Decreased post-ovulatory follicles	-	-	-	NR	NR	NR
	Vitellogenin						
	Males	+ (90.6 ng/L)	+ (100 ng/L#)	NR	NR	+	+ (100 ng/L#)
	Females	+ (90.6 ng/L)	+ (100 ng/L#)	NR	NR	+	NR
	Secondary sex characteristics						
	Males	+ (90.6 ng/L)	+ (100 ng/L#)	-	+ (100 nM#)***	NR	NR
	Females	-	-	-	-	NR	NR
	Fecundity	-	±& (100 ng/L#)	-	NR	+	NR

NR = Not reported
*Lab 4 - protocol 1 reported Sertoli cell hypertrophy/hyperplasia
**Lab 4 - protocol 1 reported increased oocyte atresia in comparison to controls in two of six treatment groups
***Lab 4 - protocol 1 reported significant atrophy of the nuptial tubercles in treated males
& = Difficult to determine if spawning status was affected
# = Nominal versus actual concentration
Labs 9 - 11 ran estradiol as a positive control for a prochloraz study, so there is only one concentration represented

4-*tert*-pentylphenol

Phase 1B studies

		Lab 9 - protocol 15 (OECD 2006)	Lab 10 - protocol 15 (OECD 2006)	Lab 11 - protocol 15 (OECD 2006)	Lab 5 - protocol 10 (Panter et al 2002)
	Core Diagnostic Criteria				
Males	Testis-ova	-	-	-	NR
	Increased proportion of spermatogonia	+ (862 µg/L)	+ (887 µg/L)	+ (820 µg/L)	NR
	Interstitial cell hypertrophy/hyperplasia	-	-	-	NR
	Increased testicular degeneration	+ (862 µg/L)	-	-	NR
Females	Increased oocyte atresia	-	-	-	NR
	Perifollicular cell hypertrophy/hyperplasia	-	-	-	NR
	Decreased yolk formation	-	-	-	NR
	Altered gonadal stage	+ (862 µg/L)	+ (887 µg/L)	-	NR
	Secondary Diagnostic Criteria				
Males	Decreased proportion of spermatogonia	-	-	-	NR
	Increased proteinaceous fluid within testicular vessels or interstitium	+ (80.1 µg/L)	-	+ (820 µg/L)	NR
	Asynchronous gonad development	-	-	-	NR
	Altered gonadal staging	+ (862 µg/L)	-	-	NR
Females	Granulomatous inflammation	-	-	-	NR
	Interstitial fibrosis	-	-	-	NR
	Egg debris in the oviduct	-	-	-	NR
	Granulomatous inflammation	-	+ (887 µg/L)	-	NR
	Decreased post-ovulatory follicles	-	-	-	NR
	Vitellogenin				
	Males	+ (270 µg/L)	+ (298 µg/L)	NR	+ (10 µg/L)
	Females	+ (862 µg/L)	-	NR	NR
	Secondary sex characteristics				
	Males	+ (↓ 862 µg/L)	+ (↓ 887 µg/L)	+ (↓ 820 µg/L)	NR
	Females	-	-	-	NR
	Fecundity	+ (↓ 862 µg/L)	+ (↓ 887 µg/L)	+ (↓ 820 µg/L)	NR

NR = not reported

Purple highlighting indicates that control groups did not spawn well

Bisphenol A

	Core Diagnostic Criteria	Lab 2 - Protocol 1 (Battelle 2004)	Lab 7 - protocol 13 (Sohoni et al 2001)
Males	Testis-ova	-	-
	Increased proportion of spermatogonia	-	+*
	Interstitial cell hypertrophy/hyperplasia	NR	-
	Increased testicular degeneration	NR	-
Females	Increased oocyte atresia	-	-
	Perifollicular cell hypertrophy/hyperplasia	NR	-
	Decreased yolk formation	NR	-
	Altered gonadal stage	-	-
	Secondary Diagnostic Criteria		
Males	Decreased proportion of spermatogonia	-	-
	Increased proteinaceous fluid within testicular vessels or interstitium	NR	-
	Asynchronous gonad development	NR	-
	Altered gonadal staging	-	-
Females	Granulomatous inflammation	-	-
	Interstitial fibrosis	NR	-
	Egg debris in the oviduct	NR	-
	Granulomatous inflammation	+ (56.6 µg/L)	-
	Decreased post-ovulatory follicles	-	-
	Vitellogenin		
	Males	+ (56.6 µg/L)	+ (640 µg/L[#])
	Females	+ (344.1 µg/L)	-
	Secondary sex characteristics		
	Males	-	NR
	Females	-	NR
	Fecundity	+ (344.1 µg/L)	NR

NR = not reported

Lab 2 - protocol 2 reported significant differences in stage 1A (increase) and stage 3 (decrease) oocytes

*It is difficult to tell when this change was significant, it may be after 164 days rather than only 42. If this is the case, then it should be considered NR for the purposes of this table.

= nominal concentration

Fadrozole, part 1 (protocols 1 & 15) – continued on next page

	Core Diagnostic Criteria	Lab 1 - protocol 1 (Ankley et al 2002)	Lab 2 - protocol 1 (Battelle 2003a)	Lab 10 - protocol 15 (OECD 2006)	Lab 11 - protocol 15 (OECD 2006)	Lab 12 - protocol 15 (OECD 2006)	Lab 13 - protocol 15 (OECD 2006)
Males	Testis-ova	NR	-	-	-	-	-
Males	Increased proportion of spermatogonia	NR	-	-	-	-	+ (104 µg/L)
Males	Interstitial cell hypertrophy/hyperplasia	NR	+ (55.7 µg/L)	-	+ (93 µg/L)	+ (97.1 µg/L)	+ (104 µg/L)
Males	Increased testicular degeneration	NR	NR	-	-	-	-
Females	Increased oocyte atresia	+ (5 µg/L[#])	+ (5.2 µg/L)	+ (100 µg/L[#])	+ (93 µg/L)	+ (97.1 µg/L)	+ (104 µg/L)
Females	Perifollicular cell hypertrophy/hyperplasia	NR	NR	-	-	-	-
Females	Decreased yolk formation	NR	NR	-	+ (93 µg/L)	-	+ (104 µg/L)
Females	Altered gonadal stage	+ (5 µg/L[#])	+ (55.7 µg/L)	+ (100 µg/L[#])	+ (93 µg/L)	+ (97.1 µg/L)	+ (104 µg/L)
	Secondary Diagnostic Criteria						
Males	Decreased proportion of spermatogonia	NR	-	-	-	-	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	-	-	-	-
Males	Asynchronous gonad development	NR	NR	-	-	-	-
Males	Altered gonadal staging	+	-	+ (100 µg/L[#])	-	-	-
Females	Granulomatous inflammation	NR	-	-	-	-	-
Females	Interstitial fibrosis	NR	NR	-	-	-	-
Females	Egg debris in the oviduct	NR	NR	+ (100 µg/L[#])	-	-	-
Females	Granulomatous inflammation	NR	-	-	-	-	-
Females	Decreased post-ovulatory follicles	+ (5 µg/L[#])	+ (55.7 µg/L)	-	-	-	-
	Vitellogenin						
	Males	-	-	-	NR	-	-
	Females	+ (↓ 2 µg/L[#])	+ (↓ 5.2 µg/L)	+ (↓ 100 µg/L[#])	NR	-	+ (↓ 104 µg/L)
	Secondary sex characteristics						
	Males	-	-	-	-	-	-
	Females	-	-	-	-	-	-
	Fecundity	+ (↓ 10 µg/L[#])	+ (↓ 55.7 µg/L)	-	NR	-	-

Orange header (Lab 2, protocol 6) indicates 14-day non-spawning assay
NR = Not reported
NA = Not applicable
Lab 1 - protocol 1 reported a significant increase in mean tubule diameter in treated males
Lab 2 - protocol 2 reported a significant increase in mean tubule diameter in treated males
= nominal versus actual concentration
Labs 10 - 13 ran fadrozole as a positive control for a prochloraz study, so there is only one concentration represented

Fadrozole, part 2 (protocols 3, 6, & 11) – continued on next page

		Lab 2 - protocol 3 (Battelle 2003a)	Lab 2 - protocol 6 (Battelle 2003a)	Lab 5 - protocol 11 (Panter et al 2004)
Core Diagnostic Criteria				
Males	Testis-ova	-	-	NR
Males	Increased proportion of spermatogonia	-	-	NR
Males	Interstitial cell hypertrophy/hyperplasia	+ (59.9 µg/L)	+ (57.0 µg/L)	NR
Females	Increased testicular degeneration	NR	NR	NR
Females	Increased oocyte atresia	+ (59.9 µg/L)	+ (57.0 µg/L)	NR
Females	Perifollicular cell hypertrophy/hyperplasia	NR	NR	NR
Females	Decreased yolk formation	NR	NR	NR
Females	Altered gonadal stage	+ (59.9 µg/L)	-	NR
Secondary Diagnostic Criteria				
Males	Decreased proportion of spermatogonia	-	-	NR
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR	NR
Males	Asynchronous gonad development	NR	NR	NR
Females	Altered gonadal staging	-	-	NR
Females	Granulomatous inflammation	-	-	NR
Females	Interstitial fibrosis	NR	NR	NR
Females	Egg debris in the oviduct	NR	NR	NR
Females	Granulomatous inflammation	-	-	NR
Females	Decreased post-ovulatory follicles	+ (5.5 µg/L)	-	NR
Vitellogenin				
Males		-	-	+ (24.8 µg/L)
Females		+ (↓ 5.5 µg/L)	+ (↓ 31.7 µg/L)	+(24.8 µg/L)
Secondary sex characteristics				
Males		-	-	+ (24.8 µg/L)*
Females		-	-	-
Fecundity		+ (↓ 59.9 µg/L)	NA	NR

Orange header (Lab 2, protocol 6) indicates 14-day non-spawning assay
NR = Not reported
NA = Not applicable
Lab 1 - protocol 1 reported a significant increase in mean tubule diameter in treated males
Lab 2 - protocol 2 reported a significant increase in mean tubule diameter in treated males
*Dose response not achieved as there was no significant difference at fadrozole levels above 24.8 µg/L.
= nominal versus actual concentration
Labs 10 - 13 ran fadrozole as a positive control for a prochloraz study, so there is only one concentration represented

Fenarimol

		Lab 1 - protocol 2 (Ankley et al 2005a)
	Core Diagnostic Criteria	
Males	Testis-ova	NR
	Increased proportion of spermatogonia	+
	Interstitial cell hypertrophy/hyperplasia	NR
	Increased testicular degeneration	NR
Females	Increased oocyte atresia	+
	Perifollicular cell hypertrophy/hyperplasia	NR
	Decreased yolk formation	NR
	Altered gonadal stage	-
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
	Increased proteinaceous fluid within testicular vessels or interstitium	NR
	Asynchronous gonad development	NR
	Altered gonadal staging	NR
	Granulomatous inflammation	NR
Females	Interstitial fibrosis	NR
	Egg debris in the oviduct	NR
	Granulomatous inflammation	NR
	Decreased post-ovulatory follicles	NR
	Vitellogenin	
	Males	-
	Females	+
	Secondary sex characteristics	
	Males	NR
	Females	NR
	Fecundity	+

NR = not reported

Ketoconazole

		Lab 1 - protocol 1 (Ankley and Villeneuve 2006)	Lab 17 - protocol 1 (Battelle 2006)
	Core Diagnostic Criteria		
Males	Testis-ova	-	-
	Increased proportion of spermatogonia	-	-
	Interstitial cell hypertrophy/hyperplasia	+	+ (18 µg/L)
	Increased testicular degeneration	-	-
Females	Increased oocyte atresia	-	-
	Perifollicular cell hypertrophy/hyperplasia	-	-
	Decreased yolk formation	-	-
	Altered gonadal stage	-	-
	Secondary Diagnostic Criteria		
Males	Decreased proportion of spermatogonia	-	-
	Increased proteinaceous fluid within testicular vessels or interstitium	-	-
	Asynchronous gonad development	-	-
	Altered gonadal staging	-	-
Females	Granulomatous inflammation	-	-
	Interstitial fibrosis	-	-
	Egg debris in the oviduct	-	-
	Granulomatous inflammation	-	-
	Decreased post-ovulatory follicles	-	-
	Vitellogenin		
	Males	-	+ (320 µg/L)
	Females	NR	-
	Secondary sex characteristics		
	Males	NR	-
	Females	NR	-
	Fecundity	+	-

NR = not reported

Prochloraz

		Lab 10 - protocol 15 (OECD 2006)	Lab 11 - protocol 15 (OECD 2006)	Lab 12 - protocol 15 (OECD 2006)	Lab 13 - protocol 15 (OECD 2006)	Lab 1 - protocol 2 (Ankley et al 2005a)
	Core Diagnostic Criteria					
Males	Testis-ova	-	-	-	-	NR
Males	Increased proportion of spermatogonia	-	-	-	+ (382 µg/L)	+
Males	Interstitial cell hypertrophy/hyperplasia	-	-	-	-	NR
Males	Increased testicular degeneration	-	-	-	-	NR
Females	Increased oocyte atresia	+ (299 µg/L)	+ (341 µg/L)	+ (275 µg/L)	+ (382 µg/L)	+
Females	Perifollicular cell hypertrophy/hyperplasia	-	-	-	-	NR
Females	Decreased yolk formation	-	-	-	-	NR
Females	Altered gonadal stage	-	+ (341 µg/L)	-	-	-
	Secondary Diagnostic Criteria					
Males	Decreased proportion of spermatogonia	-	-	-	-	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	+ (299 µg/L)	-	-	-	NR
Males	Asynchronous gonad development	-	-	-	-	NR
Males	Altered gonadal staging	-	-	-	-	+
Females	Granulomatous inflammation	-	-	-	-	NR
Females	Interstitial fibrosis	-	-	-	-	NR
Females	Egg debris in the oviduct	+ (97.5 µg/L)	-	-	-	NR
Females	Granulomatous inflammation	-	-	-	-	NR
Females	Decreased post-ovulatory follicles	-	-	-	-	NR
	Vitellogenin					
	Males	-	-	-	-	-
	Females	+ (↓ 299 µg/L)	NR	+ (↓ 275 µg/L)	+ (↓ 121 µg/L)	+
	Secondary sex characteristics					
	Males	-	-	-	-	NR
	Females	-	-	-	-	NR
	Fecundity	-	+ (↓ 341 µg/L)	-	-	+

NR = not reported

Purple highlighting indicates that there was poor spawning in the controls.

Cadmium chloride

		Lab 2 - Protocol 1 (Battelle 2004)
	Core Diagnostic Criteria	
Males	Testis-ova	-
Males	Increased proportion of spermatogonia	-
Males	Interstitial cell hypertrophy/hyperplasia	-
Males	Increased testicular degeneration	NR
Females	Increased oocyte atresia	-
Females	Perifollicular cell hypertrophy/hyperplasia	NR
Females	Decreased yolk formation	NR
Females	Altered gonadal stage	-
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR
Males	Asynchronous gonad development	NR
Males	Altered gonadal staging	-
Females	Granulomatous inflammation	NR
Females	Interstitial fibrosis	NR
Females	Egg debris in the oviduct	NR
Females	Granulomatous inflammation	NR
Females	Decreased post-ovulatory follicles	-
	Vitellogenin	
	Males	-
	Females	-
	Secondary sex characteristics	
	Males	-
	Females	-
	Fecundity	-

NR = not reported

Atrazine

		Lab 2 - protocol 1 (Battelle 2004)	Lab 3 - protocol 1 (Bringolf et al 2004)
	Core Diagnostic Criteria		
Males	Testis-ova	-	-
Males	Increased proportion of spermatogonia	+ (2A) (24.5 µg/L)	NR
Males	Interstitial cell hypertrophy/hyperplasia	-	NR
Males	Increased testicular degeneration	NR	NR
Females	Increased oocyte atresia	-	NR
Females	Perifollicular cell hypertrophy/hyperplasia	NR	NR
Females	Decreased yolk formation	NR	NR
Females	Altered gonadal stage	-	-
	Secondary Diagnostic Criteria		
Males	Decreased proportion of spermatogonia	+ (2B) (24.5 µg/L)	NR
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR
Males	Asynchronous gonad development	NR	NR
Males	Altered gonadal staging	-	NR
Females	Granulomatous inflammation	NR	NR
Females	Interstitial fibrosis	NR	NR
Females	Egg debris in the oviduct	NR	NR
Females	Granulomatous inflammation	NR	NR
Females	Decreased post-ovulatory follicles	-	NR
	Vitellogenin		
	Males	-	-
	Females	-	-
	Secondary sex characteristics		
	Males	-	-
	Females	-	-
	Fecundity	-	-

NR = not reported

Lab 2 - protocol 2 reported a significant decrease in testis tubule diameter in treated fish

PFOS

		Lab 1 - protocol 4 (Ankley et al 2005b)	Lab 8 - protocol 14 (Oakes et al 2005)
	Core Diagnostic Criteria		
Males	Testis-ova	NR	NR
Males	Increased proportion of spermatogonia	-	NR
Males	Interstitial cell hypertrophy/hyperplasia	NR	NR
Males	Increased testicular degeneration	NR	NR
Females	Increased oocyte atresia	+	NR
Females	Perifollicular cell hypertrophy/hyperplasia	NR	NR
Females	Decreased yolk formation	NR	NR
Females	Altered gonadal stage	+	NR
	Secondary Diagnostic Criteria		
Males	Decreased proportion of spermatogonia	-	NR
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR	NR
Males	Asynchronous gonad development	-	NR
Males	Altered gonadal staging	-	NR
Females	Granulomatous inflammation	NR	NR
Females	Interstitial fibrosis	NR	NR
Females	Egg debris in the oviduct	NR	NR
Females	Granulomatous inflammation	NR	NR
Females	Decreased post-ovulatory follicles	+	NR
	Vitellogenin		
	Males	-	NR
	Females	-	NR
	Secondary sex characteristics		
	Males	-	NR
	Females	-	NR
	Fecundity	+	-*

NR = not reported

*Lab 8 - protocol 1 reported trends of effects of fecundity, though they were not considered statistically significant

Prometon

		Lab 1 - protocol 2 (Ankley and Villeneuve 2006)
	Core Diagnostic Criteria	
Males	Testis-ova	-
Males	Increased proportion of spermatogonia	-
Males	Interstitial cell hypertrophy/hyperplasia	-
Males	Increased testicular degeneration	-
Females	Increased oocyte atresia	+
Females	Perifollicular cell hypertrophy/hyperplasia	-
Females	Decreased yolk formation	-
Females	Altered gonadal stage	-
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	-
Males	Asynchronous gonad development	-
Males	Altered gonadal staging	-
Females	Granulomatous inflammation	-
Females	Interstitial fibrosis	-
Females	Egg debris in the oviduct	-
Females	Granulomatous inflammation	-
Females	Decreased post-ovulatory follicles	+
	Vitellogenin	
	Males	-
	Females	-
	Secondary sex characteristics	
	Males	+
	Females	-
	Fecundity	-

NR = not reported

Potassium permanganate

		Lab 15 - protocol 1 (Battelle 2006)	Lab 17 - protocol 1 (Battelle 2006)
	Core Diagnostic Criteria		
Males	Testis-ova	-	-
Males	Increased proportion of spermatogonia	+ (389 µg/L)	+ (680 µg/L)
Males	Interstitial cell hypertrophy/hyperplasia	-	-
Males	Increased testicular degeneration	+ (389 µg/L)	-
Females	Increased oocyte atresia	+ (184 µg/L)	-
Females	Perifollicular cell hypertrophy/hyperplasia	-	-
Females	Decreased yolk formation	-	-
Females	Altered gonadal stage	+ (389 µg/L)	-
	Secondary Diagnostic Criteria		
Males	Decreased proportion of spermatogonia	-	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	-	-
Males	Asynchronous gonad development	-	-
Males	Altered gonadal staging	+ (389 µg/L)	-
Females	Granulomatous inflammation	-	-
Females	Interstitial fibrosis	-	-
Females	Egg debris in the oviduct	-	-
Females	Granulomatous inflammation	-	-
Females	Decreased post-ovulatory follicles	-	-
	Vitellogenin		
Males	Males	-	-
Females	Females	+ (↓ 389 µg/L)	-
	Secondary sex characteristics		
Males	Males	+ (850 µg/L)	-
Females	Females	-	-
	Fecundity	+ (↓ 389 µg/L)	-

NR = not reported

Perchlorate

		Lab 2 - Protocol 1 (Battelle 2006)
	Core Diagnostic Criteria	
Males	Testis-ova	-
Males	Increased proportion of spermatogonia	-
Males	Interstitial cell hypertrophy/hyperplasia	-
Males	Increased testicular degeneration	NR
Females	Increased oocyte atresia	+ (5638 µg/L)
Females	Perifollicular cell hypertrophy/hyperplasia	NR
Females	Decreased yolk formation	NR
Females	Altered gonadal stage	-*
	Secondary Diagnostic Criteria	
Males	Decreased proportion of spermatogonia	-
Males	Increased proteinaceous fluid within testicular vessels or interstitium	NR
Males	Asynchronous gonad development	NR
Males	Altered gonadal staging	-
Females	Granulomatous inflammation	-
Females	Interstitial fibrosis	NR
Females	Egg debris in the oviduct	NR
Females	Granulomatous inflammation	NR
Females	Decreased post-ovulatory follicles	-
	Vitellogenin	
Males	Males	-
Females	Females	-
	Secondary sex characteristics	
Males	Males	-
Females	Females	-
	Fecundity	-

NR = not reported
*Lab 2 - protocol 2 reported significant decreases in the proportion of stage 1A oocytes

Addendum 2

FISH SCREENING ASSAY
TEST PROTOCOL – Fathead Minnow

1.0 INTRODUCTION

The U.S. Environmental Protection Agency (US-EPA 2002) has described a short-term reproduction assay with the fathead minnow *(Pimephales promelas)* that considers reproductive fitness as an integrated measure of toxicant effects, and also enables measurement of a suite of histological and biochemical endpoints that reflect effects associated with [anti-] estrogens and androgens. The assay (Ankley et al. 2001) is initiated with mature male and female fish. During a 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics are observed, and fecundity is monitored. Assessments of fertility and F1 development can be made, if desired. At the end of the test, measurements are made of a number of endpoints reflective of the status of the reproductive endocrine system, including the gonadal-somatic index (GSI), gonadal histology, and plasma concentrations of vitellogenin.

1.1 Principle of the Assay

The experimental protocol for a short-term reproduction assay is based upon the protocol developed by Ankley et al. (2001) using the fathead minnow (*Pimephales promelas*). This assay will measure the reproductive performance of groups of fathead minnows as the primary indicator for endocrine disruption. Additional measurements of morphology, histopathology, and biochemical endpoints will be performed to ensure potential toxicological and endocrine mechanisms of concern are detected for the test chemical.

The assays will be initiated with mature male and female fish. During a 21-day chemical exposure, survival, reproductive behavior, and secondary sexual characteristics will be observed while fecundity and fertilization success will be monitored daily. At termination of the assay, measurements will be made of a number of endpoints reflective of the status of the reproductive endocrine system, including the GSI, gonadal histology, and plasma concentrations of vitellogenin.

The assays will be initiated with mature ("first time spawners") spawning adults. This will be established during a "pre-exposure" period of 14 days. The pre-exposure observation period will be used to monitor reproductive performance (semi-quantitative only) as described for the chemical exposure period.

The pre-exposure observations will occur in the same system/tanks as will be utilized for the chemical test. An overview of the tests and relevant test conditions are provided in Table 1.

Table 1. Experimental Design for the Assay Method

Parameter	**Assay Protocol**
Test species:	Reproductively active fathead minnows (4.5-6 months old)
Fish husbandry conditions:	Temp:25°C ± 1°C; D.O. >5.0 mg/L; Light: 16 h light : 8 h dark with 540 to 1080 lux; Feed: frozen brine shrimp twice daily.
Pre-exposure evaluation	Duration: 14 days; Data Collected: fecundity semi-quantitative (daily)
Dilution water	Clean, surface, well or reconstituted water
Test material	NS
Test chamber size	18 L (40 x20 x 20 cm)
Test volume:	10 L
# Exchanges/day	6 tank volume exchanges
Flow rate:	2.7 L / hr
# Concentration / chemical	3
# Replicates:	4
Weight of each fish	NS
# Fish/vessel	4 females and 2 males
Total # fish/concentration	16 females and 8 males
Feeding regime	Frozen brine shrimp, twice a day
# Controls	1 Dilution water control and a solvent control added if a solvent used
# Fish/control	4 adult females and 2 adults males per replicate = 24 fish total per exposure rate. (96 fish per test chemical)
Photo period:	16 h light : 8 h dark
Temperature:	25°C ± 1°C
Light intensity	540 - 1080 lux
Aeration:	None unless D.O. <4.9 mg/L
pH	NS
Biological endpoints:	Adult survival, reproductive behavior, secondary sexual characteristics, GSI, gonadal histology, VTG, fecundity, fertility, and plasma sex steroids (optional)
Test validity criteria:	D.O. = 60% saturation; Mean temp. 25°C ± 2°C; 90% survival in the controls and successful egg production in controls. Spawning occurs at least every 4 days in each control replicate, or approximately 15 eggs/female/day/ replicate. Fertility > 95%. Measured exposure concentration CV < 20% for all replicates.

NS = Not specified in procedure.

2.0 DESCRIPTION OF THE METHOD

2.1 Selection of Test Organisms

The exposure phase will be started with sexually dimorphic adult fish from a laboratory supply of reproductively mature animals, in spawning conditions. Based on the technical judgment of experienced laboratory personnel, fish will be reproductively mature (namely, with clear secondary sexual characteristics visible) and capable of actively spawning. For general guidance only (and not to be considered in isolation from observing the actual reproductive status of a given batch of fish), fathead minnows should be 4.5 to 6 months of age, assuming they have been cultured at 25 ± 2°C

Test fish will be selected from a laboratory population, preferably from a single stock, which has been acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test (note, this acclimation period is not an *in situ* pre-exposure period). It is strongly recommended that test fish be drawn from and in-house culture as shipping of adult fish is stressful and will interfere with reliable spawning. Fish will be fed twice per day throughout the holding period and during the exposure phase. However, fish will not be fed within 12 hours of necropsy.

Following a 48-hour settling-in period, mortalities will be recorded and the following criteria applied:

- mortalities of greater than 10% of population in seven days: reject the entire batch;
- mortalities of between 5% and 10% of population: acclimation for seven additional days; if more than 5% mortality during second seven days, reject the entire batch;
- mortalities of less than 5% of population in seven days: accept the batch.

Fish will not receive treatment for disease in the two week acclimation period preceding the test, or during the exposure period.

2.2 Water

It is well established that the fathead minnow can reproduce successfully over a wide range of water quality. Therefore, no specific water type is required for this test. Any uncontaminated surface, well, or reconstituted water in which the fish can be cultured successfully should be acceptable. The animals will be tested using a flow-through water renewal system that maintains adequate water

quality (temperature, dissolved oxygen, low ammonia, etc.), and ensures a consistent exposure to the parent chemical.

2.3 Assay System

Eighteen liter glass exposure vessels are used for the test system. As recommended by Ankley et al. (2001), dimensions of the test chambers must be such that the animals can interact in a fashion conducive to successful spawning. The test chamber contains 10 L of test solution, which is renewed once every 4 hours (6 volume exchanges per day). This particular animal loading/water renewal rate is within recommended guidelines and in studies conducted according to this method, has maintained acceptable water quality while not utilizing an excessive amount of test material.

A randomized complete block design (4 blocks with one replicate of each treatment) will be used for the reproductive assay. This design is intended to randomize out the effects associated with the local environment (i.e., light and water) and possible trends associated with the diluter during testing. All fish will be transferred from pre-validation tank and then randomly assigned a treatment within a block. The blocks are filled in a random order. Thus, when one evaluates the difference between treatment means, the variability associated with experimental environment, experimental containers, and organisms being treated is removed and only the effect of the treatment remains.

2.4 Preparation and Testing of Chemical Exposure Water

Test chemicals will possess some varied physicochemical properties that will likely require different approaches for preparation of chemical exposure water. When possible, direct addition to water will be utilized if the test chemical has sufficiently high water solubility. For test chemicals with reduced water solubility, the use of a saturator column will be required to prepare the concentrated stock solution. For difficult to test substances, a solvent may be employed as a last resort (OECD GD on difficult substances). All aqueous stock solutions will be encased in black tarpaulin during agitation and subsequent storage to prevent photo-oxidation.

2.6 Analytical Determinations

After preparation of the stock solution, determinations of the concentration will be made using appropriate methods. The concentrations of the test chemical in the exposure chambers will be measured prior to adding fish to verify that target concentrations are reached. Additionally, water samples will be removed

weekly and analyzed for the test chemicals in each test chamber (High, Med, Low, and Control, in each of four replicates).

All test chemicals will be directly measured using spectrophotometry, gas chromatography - electron capture detection (GC-ECD;), gas chromatography-mass selective detection (GC-MS;), HPLC, (high performance liquid chromatography) or ion-chromatography with conductivity detection as appropriate.

96 Hour Range-finding: A range-finding test may be necessary to establish test chemical concentrations.

The highest target test concentration for the range-finder will be based upon toxicity data for other fish species. If such information is lacking, the highest concentration will be near the solubility limit of the chemical in water. Test concentration will then be decreased by a factor of 10 for each successively lower exposure (six exposure concentrations). Range-finding tests will be performed with fish of similar age and size that will be utilized in the test. A 96-hour exposure to five test concentrations plus a control (six total), two replicates for each treatment of four females and two males per exposure tank (72 fish total). The number of mortalities that occur will be used to develop a dose response curve. Based upon the results, the highest concentration that does not result in increased mortality or signs of overt morbidity compared to controls or 1/3 the derived 96-hr LC50 will serve as the highest exposure concentration in the 21-day test.

2.7 Biochemical Determinations

Vitellogenin (VTG): Enzyme-linked immunosorbent assay (ELISA) tests will be conducted using commercially available test kits or equivalents. The methods used for the bioanalytical measurements of VTG will follow manufacturer's specifications.

Sex steroids (Optional): Plasma concentrations of 17β-estradiol, testosterone, and 11-ketotestosterone may be determined using radioimmunoassay (RIA) techniques optimized for the relatively small sample volumes obtained from the fathead minnow (Jensen *et al*. 2001)

2.8 21-day Assay Initiation and Conduct

Selection and weighing of test fish

It is important to minimize variation in weight of the fish at the beginning of the assay. For the whole batch of fish used in the test, the range in individual weights at the start of the test will be kept, if possible, within ± 20% of the arithmetic mean weight. A subsample of fish will be weighed before the test in order to estimate the mean weight.

Conditions of exposure

The test duration is 21 days.

Feeding

The fish will be fed twice per day with brine shrimp at a sufficient rate to promote active reproduction and maintain body condition. Food will be withheld from the fish for 12 hours prior to the day of sampling to aid in histology processing of small fish. Uneaten food and fecal material will be removed from the test vessels at least twice weekly, e.g., by carefully cleaning the bottom of each tank using suction. (M/TH)

Pre-exposure

The pre-exposure phase will last 14 days. The assay will use fish that are approximately 20 (+2) weeks, previously maintained in communal culture tanks. Four females and two males will be randomly assigned to the replicate exposure chambers at each treatment concentration. Additional exposure chambers may be set up for pre-exposure to account for a lack of spawning in some chambers and/or mortality during the pre-exposure spawning. Any specimens whose gender cannot be identified will be excluded from the assay. It has been reported that, at 5 to 6 months of age, males are larger and darker and exhibit nuptial tubercles, while females possess an ovipositor. The pre-exposure phase of the assay will be conducted under conditions (temperature, photo period, feeding, etc.) identical to those used during the chemical exposure. The animals will be fed frozen *Artemia* twice daily. Semi-quantitative fecundity data will be collected daily. For each assay, successful pre-exposure (suitability for testing) is established when regular spawning occurs in each test chamber every 3 to 4 days.

Chemical Exposure

After successful spawning is verified during pre-exposure as per the requirements of the assay, the chemical exposure will be initiated and continued for 21 days. The assays will be conducted at three chemical concentrations (identified in Table 2), as well as a diluent water control, with four experimental

units (replicates) per treatment. Each replicate tank will contain four female and two male fish. The test chemical will be delivered to the exposure chamber using a proportional diluter (concentrated aqueous stock solutions will be prepared without using carrier solvents). The exposure will be conducted for 21 days, during which time the appearance of the fish, behavior, and fecundity will be assessed daily. At termination of the exposure, blood samples will be removed from adults and analyzed for VTG. The gonads will also be removed for GSI determination and later histological analyses.

Frequency of analytical determinations and measurements

Prior to initiation of the exposure period, proper function of the chemical delivery system will be ensured. Additionally, all analytical methods necessary will be established, including sufficient knowledge on the substances' stability in the test system. During the test, the concentrations of the test substance will be determined at regular intervals, as follows: the flow rates of diluent and toxicant stock solution will be checked at intervals, at least twice per week, and will not vary by more than 15% throughout the test. Actual test chemical concentrations will be measured in all vessels at the start of the test and at weekly intervals thereafter.

- Results will be based on measured concentrations and will be included in reporting.
- Samples may need to be filtered (e.g., using a 0.45 μm pore size) or centrifuged. If needed, then centrifugation is the recommended procedure. However, if the test material does not adsorb to filters, filtration may also be acceptable.
- During the test, dissolved oxygen, temperature, and pH will be measured in all test vessels at least once per week. Total hardness and alkalinity will be measured in the controls and one vessel at the highest concentration at least once per week. Temperature should preferably be monitored continuously in at least one test vessel.

3.0 OBSERVATIONS AND MEASUREMENTS

3.1 Endpoints

A number of endpoints will be assessed over the course of, and/or at conclusion of the assays. A description of these endpoints (Table 3 below) and their utility, particularly in the context of the assay as an EDC screen, follows:

Survival: Daily assessment of survival will be made to provide a basis for expression and interpretation of reproductive output, that is, number of

eggs/female/day. Fish will be examined daily during the test period and any external abnormalities (such as hemorrhage, discoloration) noted. Any mortality will be recorded and the dead fish removed as soon as possible. Dead fish will not be replaced in either the control or treatment vessels.

Body Weights: Recorded at test termination.

Behavior of Adults: Abnormal behavior (relative to controls), such as hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and feeding abstinence, will be noted during the daily observations. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males, also will be noted.

Fecundity: Egg production will be determined daily. Because fathead minnows spawn within a few hours after the lights are turned on, they will not be disturbed (except for feeding) until late morning. This will allow time for spawning and fertilization to be completed and for eggs to water-harden. The spawning substrates will be removed from the tanks to enumerate any eggs that are present. Based on the published report, it is expected that one spawn typically will be composed of 50 to 250 eggs. If no embryos are present, the substrate is left in the tank; new substrates should be added to replace any that are removed. Fecundity will be expressed on the basis of surviving females per reproductive (test) day per replicate or as cumulative eggs laid over the test.

Fertilization Success: After the spawning substrate has been removed from the tank, the embryos will be carefully rolled off with a gentle circular motion of an index finger and visually inspected under appropriate magnification. If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate (number embryos/number of eggs x 100) will be easily achieved. Infertile eggs are opaque or clear with a white dot where the yolk has precipitated; viable embryos remain clear for 36 to 48 hours until reaching the eyed stage.

Appearance and observation of secondary sex characteristics: Secondary sexual characteristics are under endocrine control; therefore observations of physical appearance of the fish should be made over the course of the test, and at conclusion of the study (Appendix C). Experience to date with fathead minnows suggests that some endocrine active chemicals may initially induce changes in the following external characteristics: body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in male fathead minnow, ovipositor size in females). Notably, chemicals with certain modes of action may cause abnormal occurrence of

secondary sex characteristic in animals of the opposite sex; for example, androgen receptor agonists, such as methyltestosterone and dihydrotestosterone, can cause female fathead minnows to develop pronounced nuptial tubercles (Ankley et al. 2001; Smith 1974; and Panter et al., in press). It also has been reported that estrogen receptor agonists can decrease nuptial tubercle numbers and size of the dorsal nape pad in adult males (Miles-Richardson et al., 1999; Holbech et al., 2001). Such gross morphological observations may provide useful qualitative and quantitative information to contribute to potential future fish testing requirements.

Because some aspects of appearance (primarily color) can change quickly with handling, it is important that qualitative observations be made prior to removal of animals from the test system. Other endpoints, such as the number and size of nuptial tubercles in fathead minnow, can be quantified directly. Methods for the evaluation of secondary sex characteristics in fathead minnow are provided in Appendix C.

Humane killing of fish: At the conclusion of the exposure, the fish will be anesthetized by transfer to an oxygenated solution of MS-222 (100 mg/L buffered with 200 mg $NaHCO_3$/L) and weighed.

Blood Sampling: Blood will be collected from the caudal artery/vein (Appendix A) with a heparinized microhematocrit capillary tubule. Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 µL. Plasma is separated from the blood via centrifugation (approximately 3 minutes at 15,000 x g) and stored with protease inhibitors at -75°C to -85°C until analyzed for VTG.

Gonad Size and Histology: The first step of gonad histological analysis is necropsy and rapid gonad fixation in Davidson's fixative to prevent autolysis and cellular deterioration. Immediately after humane killing of an individual fish, length and weight measurements will be taken, collection of fresh tissues (e.g., blood) will also be performed, and gonads will be perfused with fixative (Appendix B).

After sampling the blood, the "fixed" gonads will be removed and weighed (fixed weight to the nearest 0.1 mg) to determine the GSI (GSI=100 x gonad wt/body wt). Typical GSI values for reproductively active fathead minnows range from 8 to 13% for females and from 1 to 2% for males.

The following steps will be followed for gonad fixation:

1. Using a syringe, approximately 0.5 mL of Davidson's fixative will be gently applied to the gonads *in situ*. Approximately 90 seconds following the application of fixative, the liquid fixative within the abdomen will be removed with a gauze sponge, and the gonads will be excised in a manner similar to the abdominal viscera:
    a. Using the microdissection scissors, the spermatic ducts or oviducts will be severed proximal to the genital pore.
    b. Microdissection forceps will then be applied to the spermatic ducts/oviducts. Using gentle traction, the gonads will be dissected out of the abdominal cavity in a caudal to cranial direction, severing the mesorchial/mesovarial attachments as needed using the microdissection scissors. The left and right gonads may be excised individually or they may be excised simultaneously and subsequently divided at their caudal attachment.
2. The gonads (right and left) will be placed into a pre-labeled plastic tissue cassette which will then be placed into an individual container of Davidson's fixative accompanied by the abdominal viscera. The volume of fixative in the container will be at least 20 times the approximated volume of the tissues. The fixative container will be gently agitated for 5 seconds to dislodge air bubbles from the cassette.

Using the carcass, the secondary sex characteristics will be assessed (e.g., dorsal nape pad, nuptial tubercles – Appendix C). After gonads are fixed in place and then excised, material will be placed in labeled histological cassettes. Routine histological procedures will be used to assess the condition of testes and ovaries from the fish using procedures provided in Histopathology Guidelines for Phase 1B of the OECD Fish Screening Assay for EDCs May 20, 2004. Appendix D details the post mortem and histotechnical procedures that will be used.

Vitellogenin (VTG): The measurement of VTG in plasma samples will be performed using an enzyme-linked immunoabsorbant assay (ELISA). For the ELISA, polyclonal fathead minnow (*Pimephales promelas*) VTG antibody and purified VTG protein, also from the fathead minnow, will be utilized.

Table 3 provides a summary of the measurement endpoints previously discussed. In addition, Appendix E provides an example product guide.

3.2 Statistical Methods

Descriptive statistics, including the mean, standard deviation, minimum, maximum, and quartiles, will be used to characterize each endpoint measured in the tests. Statistical significance for each endpoint and chemical will be evaluated based on the difference in the mean characteristics between the treated and control groups using analysis of variance, Tukey's multiple comparisons test, and the nonparametric Kruskal-Wallis test. Chemical-dosing regimes will be considered classifications of fixed effects (i.e., control, low dose, mid-dose, and high dose). Box plots will be used to visually characterize the effect of each treatment.

Appropriate data transformations will be applied to maintain homogeneity of the within-class variances (i.e., data expressed as a percentage may be arcsine-square-root or light transformed, counts may be square-root or log transformed, and continuous data may be transformed to the natural logarithm) (Snedecor and Cochran 1980). A rank transformation or nonparametric statistics will be used when the common data transformation is not successful in controlling heterogeneity (Daniel 1978).

Analysis may be conducted both with and without suspected outliers (Chapman et al. 1996). Potential outliers may be identified by values that exceed the median plus three times the interquartile range (i.e., the difference between the 75th and 25th percentiles). If an explanation cannot be made for the divergence of data, then both analyses will be presented, assuming that the results differ. If there are no changes to the results, then the analysis including the outliers will be presented. If differences occur, then the implications of removing the outliers will be carefully documented. If an explanation can be made for the existence of outliers, the analysis excluding outliers may be sufficient.

3.3 Performance Criteria

- Water quality characteristics will remain within the limits of tolerance described in Table 2 (water temperature did not differ by more than ± 1 □C between test vessels at any one time during the exposure period and was maintained within a range of 2 □C within the temperature ranges specified for the test species).

- There will be more than 90% survival of control animals over the duration of the chemical exposure, and the control fish in each replicate will spawn, at a minimum, every 3 to 4 days. Typically, there will be approximately 15 eggs/female/day/test chamber.

- There will be greater than 95% fertility of eggs from the control animals.

3.4 Data Reporting

Test report: The test report will include the following information:

Test substance: physical nature and relevant physical-chemical properties, chemical identification data including purity and analytical method for quantification of the test substance where appropriate, source, CAS number, lot number.

Test species: at a minimum scientific name, supplier and any pretreatment.

Test conditions: test procedure used (test type, loading rate, stocking density, etc.); method of preparation of stock solutions and flow-rate; nominal test concentrations, means of the measured values and standard deviations in test vessels and method by which these were attained and evidence that the measurements refer to the concentrations of test substance in true solution; dilution water characteristics (including pH, hardness, alkalinity, temperature, dissolved oxygen concentration, residual chlorine levels, total organic carbon, suspended solids and any other measurements made); water quality within test vessels: pH, hardness, temperature and dissolved oxygen concentration; detailed information on feeding [e.g., type of food(s), source, amount given and frequency and analyses for relevant contaminants if necessary, e.g., PCBs, PAHs and organochlorine pesticides], source and treatment of dilution water, average and ranges of water chemistry parameters, photo period, light intensity, chamber size, numbers of male and female fish per replicate, number and composition of spawning substrate, lot number of feed, number of daily water volume exchanges.

Results: evidence that controls met the validity criterion for survival, and data on mortalities occurring in any of the test concentrations; statistical analytical techniques used, statistics based on fish, treatment of data and justification of techniques used; tabulated data on biological observations of gross morphology (including secondary sex characteristics) and vitellogenin; detailed report on gonadal histology; results of the statistical analysis preferably in tabular and graphical form; incidence of any unusual reactions by the fish and any visible effects produced by the test substance; average, standard deviation, and range for each test endpoint.

Table 3. Measurement Endpoints and Associated Criteria

Parameter	Units	Expected Results
Survival: Daily assessment of survival will be made to provide a basis for expression and interpretation of reproductive output.	Not Applicable	90% or greater survival in controls. Mortality is expected to be low based on previous studies at these exposure rates.
Behavior of Adults: Abnormal behavior (relative to controls), during the daily observations will be noted.	Not Applicable	Expected observations may include: Hyperventilation, loss of equilibrium, uncoordinated swimming, atypical quiescence, and feeding abstinence. Alterations in reproductive behavior, particularly loss of territorial aggressiveness by males. Qualitative anecdotal observations.
Fecundity: Egg production will be determined daily, but only during the morning.	Fecundity will be expressed either on the basis of average number of eggs laid by surviving females per reproductive (test) day per replicate or as cumulative eggs laid over the test.	It is expected that one spawn typically will be composed of 10 to 250 eggs. If no embryos are present, the substrate will be left in the tank; new substrates should be added to replace any that are removed.
Fertilization Success: If spawning occurred that morning, embryos typically will be undergoing late cleavage, and determination of the fertility rate is easily achieved.	Number embryos/number of eggs x 100	Fertilized eggs will be apparent within a few hours of fertilization. Infertile eggs will be opaque or clear with a white dot where the yolk has precipitated. Control fertilization should be ≥95%.

Parameter	Units	Expected Results
Appearance of Adults: The external appearance of the adults will be assessed as part of the daily observations, and any unusual changes will be noted. These observations are especially important for assessing endocrine active agents that are (anti)-androgenic.	Not Applicable	External features of particular importance include body color (light or dark), coloration patterns (presence of vertical bands), body shape (head and pectoral region), and specialized secondary sex characteristics (size of dorsal nape pad, number of nuptial tubercles in males; ovipositor size in females).
Body Weights	Grams	Normal/increased/decreased relative weights to control animals.
Blood Samples: will be collected from the caudal artery/vein with a heparinized microhematocrit capillary tubule and analyzed for VTG	Depending upon the size of the fathead minnow (which usually is sex-dependent), blood volumes generally range from 30 to 80 µL.	Plasma will be separated from the blood sample via centrifugation (approx. 3 minutes at 15,000 x g) and stored with protease inhibitors at -75°C to -85°C until analysis.
Vitellogenin (VTG) Concentration	pg/mL	The measurement of VTG in plasma samples will be performed using an enzyme-linked immunosorbent assay (ELISA). For the ELISA, polyclonal Fathead minnow (FHM) *(Pimephales promelas)* VTG antibody and purified VTG protein, also from the FHM, will be utilized.
Gonad Size: After sampling the blood, the fixed gonads removed and weighed (to the nearest 0.1 mg) to determine the GSI (GSI=100 x gonad wt/body wt).	Not Applicable	Typical GSI values for reproductively active fathead minnows range from 8 to 13% for females and from 1 to 2% for males. Many chemicals that reduce fecundity also will reduce the GSI in one or both sexes.
Gonad Morphology: Routine histological procedures will be used to assess the condition of testes and ovaries from the fish. Gonads will be placed in fixative (Davidson's fixative). EPL will perform histology procedures and will follow the protocol from the OECD Phase 1B Study.	Not Applicable	Evaluation of the testis will be based on the amount of germinal epithelium present and the degree of spermatogenic activity. The ovary will be evaluated based upon relative numbers of perinucleolar, cortical alveolar, and vitellogenic oocytes.

Not Applicable. No unit can be defined for this parameter.

4.0 CONTINGENCIES

The three problems most likely to be encountered are related to insufficient numbers of spawning tanks that are successfully "pre-validated," unplanned mortality in control or exposure tanks, and low or high measured concentrations relative to the nominal level of the test chemicals. These problems will be dealt with in the following manner:

- For each chemical, extra spawning tanks (up to 8 additional tanks) will be pre-validated to ensure an adequate supply of spawning fathead minnows.

- If there is excessive unscheduled mortality in any tank, the experiment should be terminated and the cause investigated.

- Prior to initiation of the chemical exposure, each diluter will be tested for up to 1 week for its ability to maintain the desired concentration. If, during the exposures the measured concentration becomes unacceptably low or high, adjustments will be made to the diluter to correct the problem.

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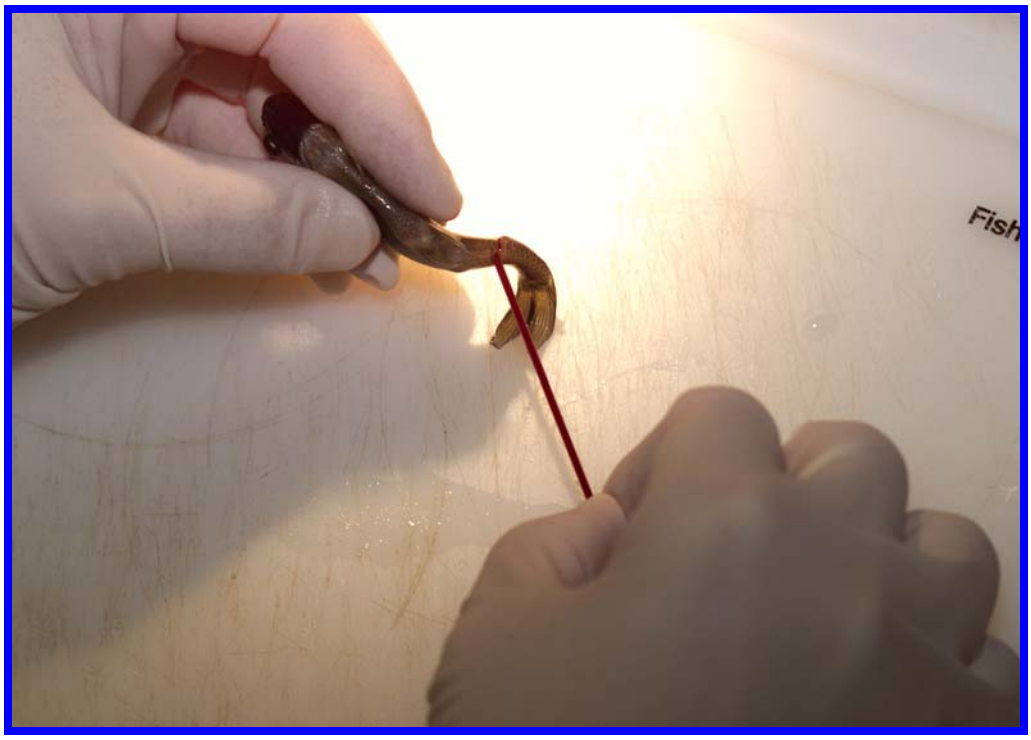
APPENDIX A

SAMPLE COLLECTION PROCEDURES FOR VITELLOGENIN ANALYSIS

Procedure 1A: Fathead Minnow, Blood Collection from the Caudal Vein/Artery



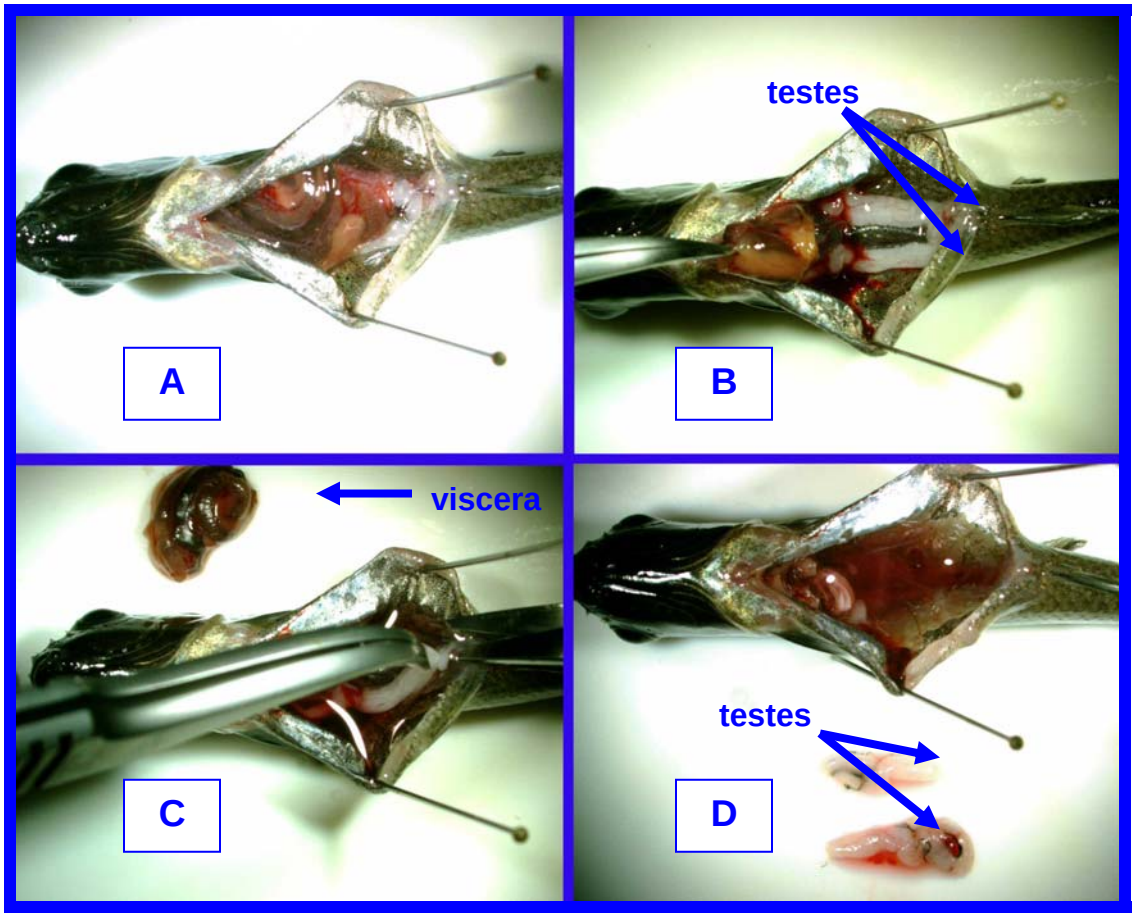
After anaesthetization, the caudal peduncle is partially severed with a scalpel blade and blood is collected from the caudal vein/artery with a heparinized microhematocrit capillary tube. After the blood has been collected, the plasma is quickly isolated by centrifugation for 3 min at 15,000 *g*. If desired, percent hematocrit can be determined following centrifugation. The plasma portion is then removed from the microhematocrit tube and stored in a centrifuge tube with 0.13 units of aprotinin (a protease inhibitor) at -75°C to -85°C until determination of vitellogenin and sex steroid concentrations can be made. Depending on the size of the fathead minnow (which is sex-dependent), collectable plasma volumes generally range from 20 to 60 microliters per fish (Jensen *et al.* 2001).

Procedure 1B: Fathead Minnow, Blood Collection from Heart

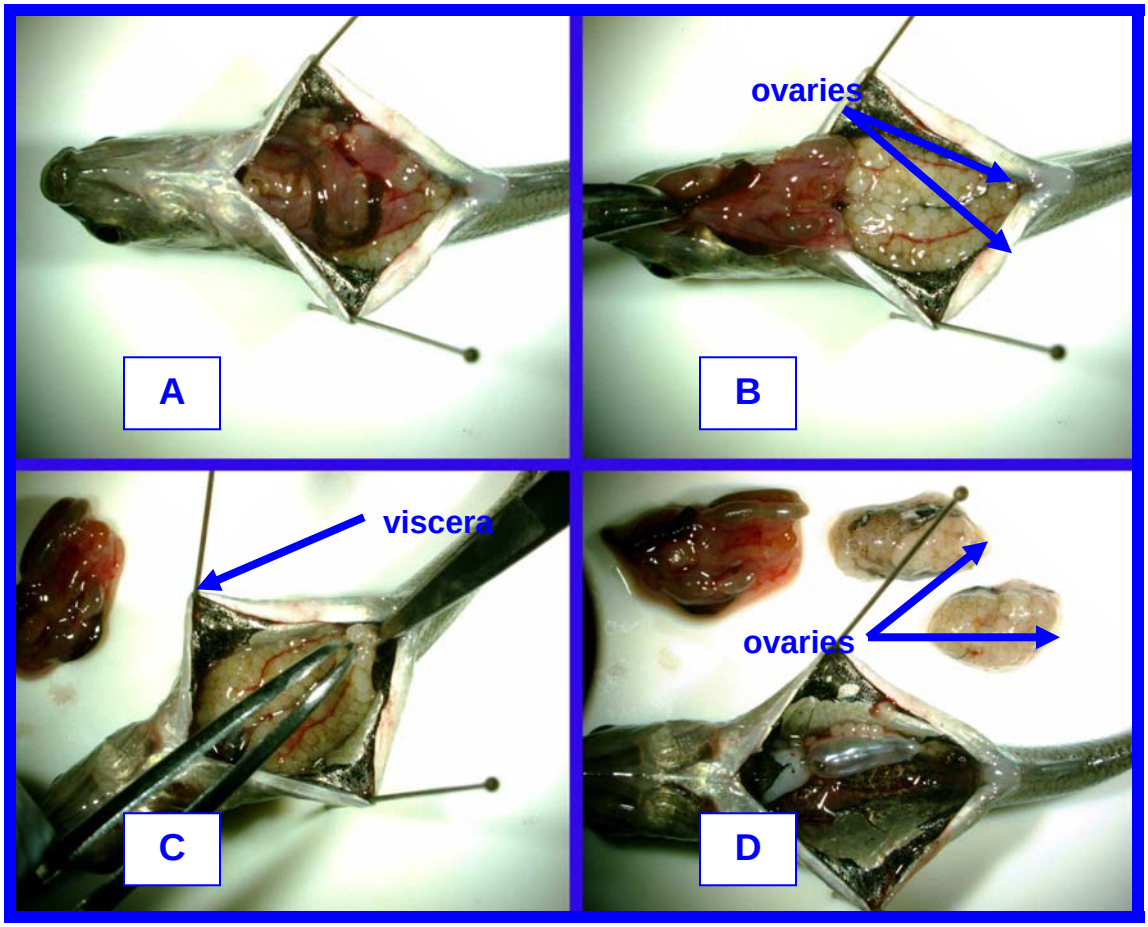
Alternatively, blood may also be collected by cardiac puncture using a heparinized syringe (1000 units of heparin per ml). The blood is transferred into Eppendorf tubes (held on ice) and then centrifuged (5 min, 7,000 g, room temperature). The plasma should be transferred into clean Eppendorf tubes (in aliquots if the volume of plasma makes this feasible) and promptly frozen at -75°C to -85°C, until analyzed (Panter et al., 1998).

APPENDIX B

Removal of gonads from Fathead minnows



Fathead Minnow, Male: Excision of the testes during necropsy.
A. The abdominal wall is pinned laterally. B. The terminal intestine is severed and retracted prior to removal. C. The testes are grasped near the spermatic ducts. D. Removal of the testes is complete.



Fathead Minnow, Female: Excision of the ovaries during necropsy. A. The abdominal wall is pinned laterally. B. The terminal intestine is severed and retracted prior to removal. C. The ovaries are grasped near the oviducts. D. Removal of the ovaries is complete.

APPENDIX C

Assessment of Secondary Sex Characteristics (Nuptial Tubercles) in EDC Tests with Fathead Minnows

Michael Kahl and Gerald Ankley

Overview

Potentially important characteristics of physical appearance in adult fathead minnows in endocrine disrupter testing include body color (i.e., light/dark), coloration patterns (i.e., presence or absence of vertical bands), body shape (i.e., shape of head and pectoral region, distension of abdomen), and specialized secondary sex characteristics (i.e., number and size of nuptial tubercles, size of dorsal pad and ovipositor).

Nuptial tubercles are located on the head (dorsal pad) of reproductively-active male fathead minnows, and are usually arranged in a bilaterally-symmetric pattern (Jensen et al. 2001). Control females and juvenile males and females exhibit no tubercle development (Jensen et al. 2001). There can be up to eight individual tubercles around the eyes and between the nares of the males. The greatest numbers and largest tubercles are located in two parallel lines immediately below the nares and above the mouth. In many fish there are groups of tubercles below the lower jaw; those closest to the mouth generally occur as a single pair, while the more ventral set can be comprised of up to four tubercles. The actual numbers of tubercles is seldom more than 30 (range, 18-28; Jensen et al. 2001). The predominant tubercles (in terms of numbers) are present as a single, relatively round structure, with the height approximately equivalent to the radius. Most reproductively-active males also have, at least some, tubercles which are enlarged and pronounced such that they are indistinguishable as individual structures.

Some types of endocrine-disrupting chemicals (EDCs) can cause the abnormal occurrence of certain secondary sex characteristics in the opposite sex; for example, androgen receptor agonists, such as 17α-methyltestosterone or 17β-trenbolone, can cause female fathead minnows to develop nuptial tubercles (Smith 1974; Ankley *et al.* 2001; 2003), while estrogen receptor agonists may decrease number or size of nuptial tubercles in males (Miles-Richardson et al. 1999; Harries et al. 2000).

This protocol describes characterization of nuptial tubercles in fathead minnows based on procedures used at the U.S. Environmental Protection Agency lab in Duluth, MN. Specific products and/or equipment can be substituted with comparable materials available in participating labs.

Protocol

Anesthetic

MS-222 is used as an anesthetic for fish sampling/assessment. Sodium bicarbonate is used as a buffering agent for the sedative.

Reagents:
MS-222 - Fenquel™ (Tricaine Methanesulfonate, Argent Chemical Laboratories, Redmond, WA 98052, USA).

Sodium bicarbonate - $NaHCO_3$, (J.T. Baker Inc., Phillipsburg, NJ 08865, USA).

Procedure:
1. Collect 1 L of control test water at nominal test temperature (e.g., 25°C) in a beaker
-allocate 100 mg of MS-222 to weigh pan
-allocate 200 mg of sodium bicarbonate to weigh pan
Add weighed chemicals to control water and stir (ca., 1 minute)
Transfer dissolved chemical solution to stainless steel bowl for easy fish handling
Solution will accommodate 20 to 30 organisms (added individually); fresh solution will need to be prepared for additional animals

Sampling Methods
Procedure:
1. Using a 12.5cmX10cm (125mm) fine mesh nylon net, carefully net organism from culture or test chamber.
- If handling toxicant-exposed fish, start with control fish and work up with increasing EDC concentrations.
2. Place organisms in MS-222 solution.
- Activity level may be momentarily high with rapid swimming or darting. Activity will decrease but gill ventilation rate may become elevated or rapid.
3. Within about 1 minute fish will start to show loss of equilibrium.
- Spiral or erratic swimming.
- Loss of movement, listlessness.
- Gentle probing with the net will cause little physical response. Organisms are still actively ventilating.
4. Remove fish from anesthetic with a net. Wipe excess moisture from net and fish into an absorbent towel. Gently place fish on petri dish - lack of movement occurs.
- Fish should not be actively moving; muscle tissue should still be rigid without loss of character. Continued emersion into MS-222 may be required. If potency of MS-222 is not adequate, additional chemical
($\leq$ 10 mg) may be added to strengthen effectiveness.
5. Viewing is best accomplished using an illuminated magnifying glass or 3X illuminated dissection scope. View fish dorsally and anterior forward (head toward viewer).
a. Place fish in small petri dish (e.g., 100 mm in diameter), anterior forward, ventral side down. Focus viewfinder to allow identification of tubercles. Gently and slowly roll fish from side to side to identify tubercle areas. Count and score tubercles.
b. Repeat the observation on the ventral head surface by placing the fish dorsal anterior forward in the petri dish.
c. Observations should be completed within 2 min for each fish.
d. Return fish to control water to revive, if desired.

6. If fish are handled in a gentle manner within a reasonable amount of time during tubercle assessment recovery will occur within a few minutes without lasting adverse effects. To avoid mortality during and after this procedure be alert to the following details.
- Keep fish moist during procedure.
- Limit the amount of time used to score tubercles.
- When placing fish into clean water gently move the fish back and forth, aiding water movement across the gill membranes.

7. If tubercles are assessed at test conclusion, animal may be subjected to additional sampling at this time (e.g., removal of blood for vitellogenin measurements; dissection of gonads).

Tubercle Counting and Rating

Six specific areas have been identified for assessment of tubercle presence and development in adult fathead minnows. A template was developed to map the location and quantity of tubercles present (attachment 1). The number of tubercles is recorded and their size can be quantitatively ranked as: 1-present, 2-enlarged and 3-pronounced for each organism (Fig. 1).

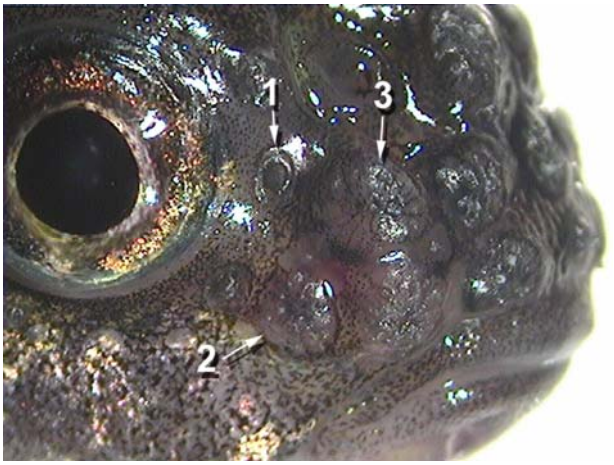


Figure1. The actual number of tubercles in some fish may be greater than the template boxes (Attachment 1) for a particular rating area. If this happens, additional rating numbers may be marked within, to the right or to the left of the box. The template therefore does not have to display symmetry. An additional technique for mapping tubercles which are paired or joined vertically along the horizontal plane of the mouth could be done by double-marking two tubercle rating points in a single box.

Rating 1-present, is identified as any tubercle having a single point whose height is nearly equivalent to its radius (diameter). Rating 2- enlarged; identified by tissue resembling an asterisk in appearance; usually has a large radial base with grooves or furrows emerging from the center. Tubercle height is often more jagged but can be somewhat rounded at times. Rating 3- pronounced; usually quite large and rounded with less definition in structure. At times these tubercles will run together forming a single mass along an individual or combination of areas (B, C and D, described below). Coloration and design are similar to rating 2 but at times are fairly indiscriminate. Using this rating system generally will result in overall tubercle scores of <50 in a normal control male possessing a tubercle count of 18 to 20 (Jensen et al. 2001).

Mapping regions:

A - Tubercles located around eye. Mapped dorsal to ventral around anterior rim of eye. Commonly multiple in mature control males, not present in control females, generally paired (one near each eye) or single in females exposed to androgens.

B - Tubercles located between nares, (sensory canal pores). Normally in pairs for control males at more elevated levels (2- enlarged or 3- pronounced) of development. Not present in control females with some occurrence and development in females exposed to androgens.

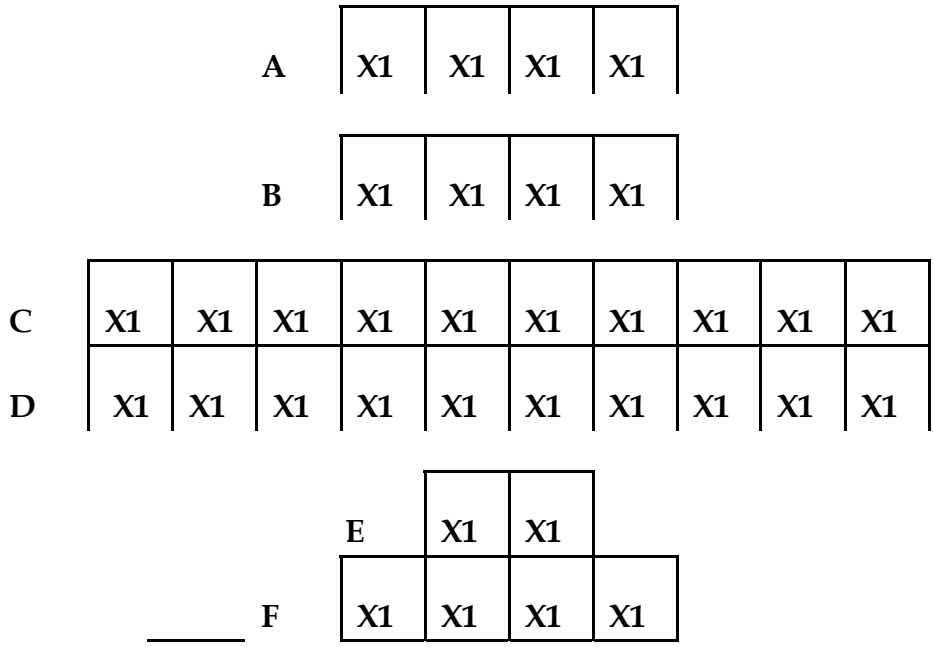
C - Tubercles located immediately anterior to nares, parallel to mouth. Generally enlarged or pronounced in mature control males. Present or enlarged in less developed males or androgen-treated females.

D - Tubercles located parallel along mouth line. Generally rated developed in control males. Absent in control females but present in androgen-exposed females.

E - Tubercles located on lower jaw, close to mouth, usually small and commonly in pairs. Varying in control or treated males, and treated females.

F - Tubercles located ventral to E. Commonly small and paired. Present in control males and androgen-exposed females.

ID_____ **Tubercle Template** **Numerical Rating**
Date_____ 1-present
Total Score_____ 2-enlarged
3-pronounced



Fatpad Score

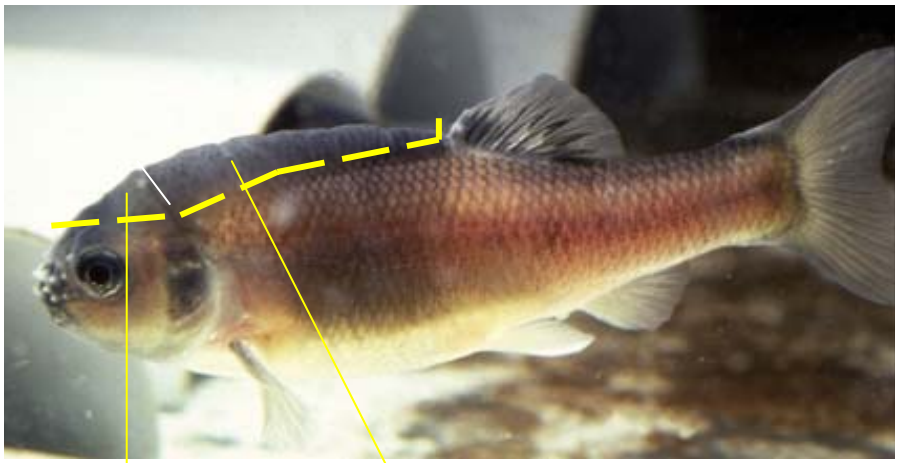
For evaluating the fatpads, use a very similar system as to that used in tubercle assessment:

1 No fatpad visible
2 Small fatpad evident
3 Fatpad is clearly visible and is just above body surface
4 Fatpad is prominent, and is clearly above the body surface, but not 'overhanging'
5 Fatpad is very prominent and is starting to 'overhang' the body surface

These evaluations are rather sufficient to identify chemical effects during exposure and should be accompanied by fatpad removal at the end of the experiment for a more accurate assessment of the fatpad.

Fatpad Index (based on personal communication from Karen Thorpe)

The fatpad index is expressed as a percentage of the body weight, i.e. Fatpad index = fatpad weight/total wet body weight.



Fatpad Index (FPI) – score around the edge of the fatpad using a scalpel and then starting from the dorsal fin and working towards the head, gently peel the fatpad away from the dorsal musculature. Be careful not to remove the muscle with the fatpad, as this will affect the overall weight. The fatpad consists of two regions, the head pad and the dorsal pad. Once you reach the head pad, stop, and then starting from the head and working back to the dorsal pad carefully slice of the head pad to the point where it is attached to the dorsal pad. Carefully sever any points at which the fatpad is still attached to the body of the fish. Weigh the fatpad.
Fatpad index (FPI) = (fatpad weight (mg)/total wet body weight (mg))*100

APPENDIX D

I. POST-MORTEM AND HISTOTECHNICAL PROCEDURES

The purpose of this section is to outline all of the post-mortem steps and procedures that occur prior to the evaluation of histologic sections on glass slides, to include euthanasia, necropsy, tissue fixation.

Post-mortem procedures:

1. Substrate obtained for vitellogenin analysis.
    a. FHM: blood sample from the caudal vein/artery or heart
2. Tissue specimen for gonad histopathology. For each species, a technique was selected that would most optimally: 1) preserve the cellular structure of the gonads; 2) maximize the amount of gonad tissue available for analysis; 3) sample the gonads in a representative and consistent fashion; and 4) allow the pathologist to examine at least three step sections of both gonads on a single glass slide.
    a. FHM: gonads excised from fish.

Davidson's fixative was selected as the recommended fixative. Compared to other common fixatives such as 10% neutral buffered formalin or Bouin's fixative, the advantages of Davidson's fixative are: 1) the morphologic appearance of gonad sections is generally considered to be comparable to sections fixed in Bouin's fixative and superior to sections fixed in formalin; 2) compared to Bouin's fixative, which contains picric acid, Davidson's fixative is generally considered to be less noxious, less hazardous, and more easily disposed of; 3) there is anecdotal information which suggests that Bouin's fixative may be difficult to obtain in the near future; 4) specimens fixed in Bouin's fixative require multiple rinses prior to transfer to alcohol or formalin. Please see photographic comparison of specimens fixed in Davidson's versus Bouin's fixatives (Histology Figures 1 provided below). Please be aware that different recipes and products that are designated as "Davidson's fixative" may actually be modifications of the original formula if a modified Davidson's fixative is used, this should be noted by the participating laboratory. Davidson's recipe is provided below:

Davidson's Fixative

Formaldehyde (37-40%)	200 ml
Glycerol	100 ml
Glacial acetic acid	100 ml
Absolute alcohol	300 ml
Distilled water	300 ml

Modified Davidson's Fixative

Formaldehyde (37-40%)	220 ml
Glacial acetic acid	115 ml
95% Ethyl alcohol	330 ml
Distilled water	335 ml

HISTOLOGY FIGURES 1

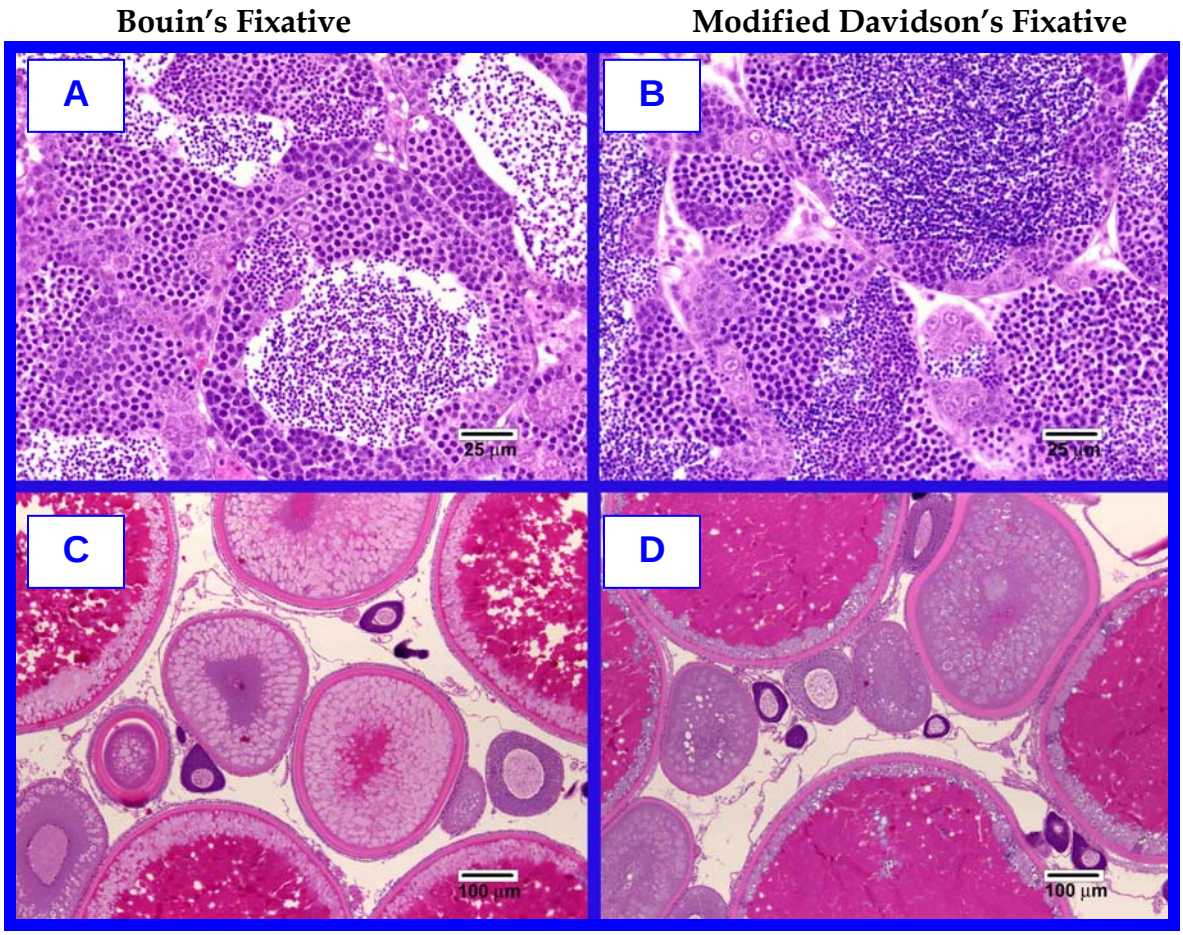


Fig. 1. Fathead Minnows, Testis (A&B) and Ovary (C&D): Gonads fixed in Bouin's fixative (A&C) and modified Davidson's fixative (B&D). Color contrast was slightly superior in testes fixed with Davidson's fixative and was clearly superior in ovaries fixed with Bouin's fixative. Either fixative is satisfactory for diagnostic purposes; however, Davidson's fixative was selected for the Phase 1B assay.

Fathead Minnow

1. Euthanasia, Necropsy, and Tissue Fixation

Objectives:

1. Provide for the humane sacrifice of fish.
2. Obtain necessary body weights and measurements.
3. Obtain specimens for vitellogenin analysis.
4. Excise gonad specimens.
5. Evaluate secondary sex characteristics.
6. Provide for adequate fixation of the gonads and the carcass.

Materials:

1. Fish transport container (Approx. ~500 ml, contains water from the experimental tank or system reservoir).
2. Small dip net.
3. Euthanasia chamber (Approx. ~500 ml vessel).
4. Euthanasia solution
5. Electronic slide caliper (minimum display: ≤ 0.1mm)
6. Electronic analytical balance (minimum display: ≤ 0.1mg) and tared vessels.
7. Stereoscopic microscope.
8. Pins and corkboard.
9. Small scissors (e.g., iris scissors).
10. Small forceps.
11. Microdissection forceps.
12. Microdissection scissors.
13. Gauze sponges.
14. Davidson's fixative
15. Plastic syringe (3ml).
16. Standard plastic tissue cassettes (one per fish).
17. Fixation containers (100 ml, one per fish).

Procedures:

1. Fish should be sacrificed within one to two minutes prior to necropsy. Therefore, unless multiple prosectors are available, multiple fish should not be sacrificed simultaneously.

2. Using the small dip net, a fish is removed from the experimental chamber and transported to the necropsy area in the transport container. For each test chamber, all male fish are sacrificed prior to the sacrifice of female fish; the sex of each fish is determined by external body characteristics (e.g., presence or absence of nuptial tubercles, dorsal pad, etc.).

3. The fish is placed into the euthanasia solution. The fish is removed from the solution when there is cessation of respiration and the fish is unresponsive to external stimuli.

4. The fish is wet weighed, measured according to protocol, and a blood sample is obtained from the caudal artery/vein or heart .
5. The fish is placed on a corkboard on the stage of a dissecting microscope. Using iris scissors and small forceps, the abdomen is opened via a carefully made incision that extends along the ventral midline from the pectoral girdle to a point just cranial to the anus.

6. The fish is placed in dorsal recumbency and the opposing flaps of body wall are pinned laterally to expose the abdominal viscera.
7. Using the small forceps and small scissors, the abdominal viscera (liver, gastrointestinal tract, spleen, pancreatic tissue, and abdominal mesentery) are carefully removed *en masse* in the following manner:
    a. The intestine is severed proximal to the anus.
    b. A forceps is applied to the terminal portion of the intestine. Using gentle traction and taking care not to disturb the gonads, the viscera are dissected out of the abdominal cavity in a caudal to cranial direction.
    c. The distal esophagus is severed just proximal to the liver.
8. Using a syringe, approximately 0.5 ml of Davidson's fixative is then gently applied to the gonads *in situ*. Approximately 90 seconds following the application of fixative, the liquid fixative within the abdomen is removed with a gauze sponge, and the gonads are excised in a manner similar to the abdominal viscera:
    a. Using the microdissection scissors, the spermatic ducts or oviducts are severed proximal to the genital pore.
    b. Microdissection forceps are then applied to the spermatic ducts/oviducts. Using gentle traction, the gonads are dissected out of the abdominal cavity in a caudal to cranial direction, severing the mesorchial/mesovarial attachments as needed using the microdissection scissors. The left and right gonads may be excised individually or they may be excised simultaneously and subsequently divided at their caudal attachment.
9. The gonads (right and left) are placed into a pre-labeled plastic tissue cassette which is then placed into an individual container of Davidson's fixative accompanied by the abdominal viscera. The volume of fixative in the container should be at least 10 times the approximated volume of the tissues. The fixative container is gently agitated for five seconds to dislodge air bubbles from the cassette.
10. Using the carcass, the secondary sex characteristics are assessed (e.g., dorsal nape pad, nuptial tubercles

11. All tissues remain in Davidson's fixative overnight, followed by transfer to individual containers of 10% neutral buffered formalin the next day. Containers with cassettes are gently agitated for 5 seconds to ensure adequate penetration of formalin into cassettes (it is not necessary to rinse with water or perform multiple changes in formalin).